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<b>(54) Title:</b> ADENOVIRUS VECTORS SPECIFIC FOR CELLS EXPRESSING ANDROGEN RECEPTOR AND METHODS OF USE THEREOF  <b>(57) Abstract</b> <p>Replication-competent adenovirus vectors specific for cells which allow a probasin transcriptional response element (PB-TRE) to function, such as cells which express the androgen receptor (AR), and methods of use of such viruses are provided. These viruses comprise an adenoviral gene under control of a transcriptional regulatory portion of a PB-TRE, which is in turn dependent upon AR expression. The gene can be, for example, a gene required for viral replication or the adenovirus death protein gene (ADP). The viruses can also comprise at least one additional adenoviral gene under control of at least one additional prostate-specific transcriptional response element, such as that controlling prostate-specific antigen expression (PSA-TRE). Thus, virus replication can be restricted to target cells exhibiting prostate-specific gene expression, particularly prostate carcinoma cells. An adenovirus of the present invention can further comprise a heterologous gene such as a reporter under transcriptional control of a PB-TRE. The adenovirus vectors can be used to detect and monitor samples for the presence of prostate cells as well as to selectively kill malignant cells producing prostate-specific gene products.</p>			

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**ADENOVIRUS VECTORS SPECIFIC FOR CELLS EXPRESSING ANDROGEN  
RECEPTOR AND METHODS OF USE THEREOF**

5                   CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Patent Application Serial No. 60/039,762, filed on March 3, 1997.

10                   STATEMENT OF RIGHTS TO INVENTIONS  
MADE UNDER FEDERALLY SPONSORED RESEARCH  
(Not applicable)

15                   TECHNICAL FIELD

This invention relates to cell transfection using adenoviral vectors, especially replication-competent adenoviruses, and methods of their use. More specifically, it relates to cell-specific replication of adenovirus vectors in cells expressing the androgen receptor, particularly prostate carcinoma cells, through use of a probasin transcriptional regulatory  
20    element.

**BACKGROUND OF THE INVENTION**

There are three significant diseases of the prostate: benign prostate hyperplasia  
25    (BPH), prostate cancer, and prostatitis. The cost of treating these three diseases is immense. The annual treatment of prostate diseases in the U.S. required 4.4 million physician visits, 836,000 hospitalizations, and cost over \$3 billion in 1985. Approximately one out of every four males above the age of 55 suffers from a prostate disease of some form or another. Prostate cancer is the fastest growing cause of cancer in men, with  
30    approximately 244,000 new cases diagnosed and about 44,000 deaths reported for 1995 in

the United States. Due to the aging U.S. population, the incidence of BPH and prostate cancer is likely to increase.

BPH causes urinary obstruction resulting in urinary incontinence. It occurs in almost 80% of men by the age of 80. Unregulated dihydrotestosterone is believed to cause hyperplastic prostate growth. Pharmacotherapy for the treatment of BPH is currently aimed at relaxing prostate smooth muscle (alpha blockade) and decreasing prostate volume (androgen suppression). Phase III clinical trials are underway to evaluate selective alpha<sub>1</sub> blockers, antiandrogens, and 5-alpha reductase inhibitors for the treatment of BPH. The most promising of these is finasteride, which has shown an ability to cause regression of the hyperplastic prostate gland in a majority of patients. Mocellini et. al. (1993) *Prostate* 22:291.

BPH is treated surgically with a transurethral resection of the prostate (TURP). This procedure is very common: 500,000 TURPs are performed in the U.S. each year and 25% of men will require surgery at some time in their lives to alleviate urinary obstruction. This makes BPH the second most common cause of surgery in males. The TURP procedure requires several days of hospitalization as well as the surgery itself. The average medical reimbursement cost of a TURP in 1987 dollars was \$8,000; in 1993 dollars this is \$14,000. Unfortunately, a side-effect of the TURP is the elimination of the ejaculatory ducts as well as the nerve bundles of the penis, resulting in impotence in 90% of patients. A TURP is prefaced by an outpatient biopsy procedure to determine if the enlargement of the prostate is benign or cancerous, which also adds to the cost. Hypertrophy may also be treated by transurethral insertion of a tubular stent or expandable dilation catheter to maintain the patency of the urethral lumen. U.S. Patent No. 4,893,623, issued January 16, 1990, to Rosenbluth et al.; and U.S. Patent No. 5,527,336, issued June 18, 1996, to Rosenbluth et al.

An alternative therapy for prostate diseases involves radiation therapy. A catheter has been developed which squeezes prostate tissue during microwave irradiation; this increases the therapeutic temperature to which the prostate tissue more distal to the microwave antennae can be heated without excessively heating nearby non-prostate tissue. U.S. Patent No. 5,007,437, issued April 16, 1991, to Sterzer et al. A combination of a

radiating energy device integrated with a urinary drainage Foley type catheter has also been developed. U.S. Patent No. 5,344,435, issued September 6, 1994, to Turner et al.

Prostate cancer is now the most frequently diagnosed cancer in men. Prostate cancer is latent; many men carry prostate cancer cells without overt signs of disease.

- 5 Autopsies of individuals dying of other causes show prostate cancer cells in 30% of men at age 50; by age 80, the prevalence is 60%. Further, prostate cancer can take up to 10 years to kill the patient after initial diagnosis. Prostate cancer is newly diagnosed in slightly over 100,000 men in the U.S. each year, of which over 40,000 will die of the disease. There is also high morbidity. Cancer metastasis to bone (late stage) is common and often
- 10 associated with uncontrollable pain. Metastasis also occurs to lymph nodes (early stage).

- The disease progresses from a well-defined mass within the prostate, to a breakdown and invasion of the lateral margins of the prostate, to metastasis to regional lymph nodes, to metastasis to the bone marrow. The aggressiveness of prostate tumors varies widely. Some tumors are relatively aggressive, doubling every six months, whereas
- 15 other are extremely slow-growing, doubling once every five years. As a consequence of the slow growth rate, few cancer cells are actively dividing at any one time. As a result, prostate cancer is generally resistant to radiation and chemotherapy, although both therapeutic modalities are widely used. Surgery is the mainstay of treatment but it too is largely ineffective and also removes the ejaculatory ducts, resulting in impotence.

- 20 Unfortunately, in 80% of cases, diagnosis of prostate cancer is established when the disease has already metastasized to the bones. Of special interest is the observation that prostate cancers frequently grow more rapidly in sites of metastasis than within the prostate itself, the site of the primary cancer.

- At this stage there is no effective cytotoxic chemotherapy for prostate cancer.
- 25 Current therapeutic techniques include the use of chemical forms of medical castration by shutting down androgen production in the testes, or directly blocking androgen production in the prostate. For the treatment of prostate cancer oral estrogens and luteinizing releasing hormone analogs are used as well as surgical removal of glands that produce androgens (orchiectomy or adrenalectomy). However, estrogens are no longer recommended because
- 30 of serious, even lethal, cardiovascular complications. Luteinizing hormone releasing hormone (LHRH) analogs are used instead. However, hormonal therapy invariably fails

with time with the development of hormone-resistant tumor cells. It is not known whether these cells develop as a mutation of the original hormone sensitive cells, or a separate class of cells. Furthermore, since 20% of patients fail to respond to hormonal therapy, it is believed that hormone-resistant cells are present at the onset of therapy.

5 Estramustine, a steroidal nitrogen mustard derivative, was originally thought to be suitable for targeted drug delivery through conjugation of estrogen to toxic nitrogen mustard. Clinical trials, however, have been disappointing when survival is used as an endpoint. Finasteride, a 4-aza steroid (Proscar® from Merck & Co.), inhibits the enzyme responsible for the intracellular conversion of testosterone to dihydrotestosterone, the most  
10 potent androgen in the prostate. Casodex® is thought to inhibit cellular uptake of testosterone by blocking androgen receptors in the nucleus. However, almost all advanced cancer prostate cells fail to respond to androgen deprivation. Indolocarbazole derivatives such as K-252a have also recently been developed to treat prostate diseases. U.S. Patent No. 5,516,771, issued May 14, 1996, to Dionne.

15 None of these techniques for treating prostate diseases has been universally successful. Following localized therapy, up to 40% of patients with advanced disease, and a large proportion of all patients, eventually develop metastatic disease. Treatment for advanced disease initially involving hormonal manipulations and palliative radiotherapy have demonstrated symptomatic relief, but not long-term disease-free survival. The use of  
20 cytotoxic agents in the management of hormone-resistant advanced prostate cancer remains poorly defined. A few single agents have become "standard therapy", although demonstration of their efficacy, by contemporary standards, is lacking. Combination chemotherapy is frequently employed, although its contribution to overall patient management is largely unsubstantiated, especially when critical assessment of efficacy  
25 parameters are used. Newer approaches using chemohormonal therapy and hormonal priming therapies have failed. High-dose chemotherapy with transplant regimens are not well-tolerated in an elderly population, to which most victims of prostate cancer belong. A growth factor inhibitor, suramin, has shown promising initial results. However, no therapy to date has been demonstrated to improve overall survival in patients with advanced  
30 hormone refractory prostate cancer. U.S. Patent No. 5,569,667, issued October 29, 1996, to Grove et al.

A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells, while leaving unaffected the function of normal cells. Thus, the therapeutic ratio, or ratio of tumor cell killing to normal cell killing of traditional tumor chemotherapy, is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and prophylaxis of prostatic hyperplasia and neoplasia are needed.

Of particular interest is development of more specific, targeted forms of therapy for prostate diseases. In contrast to conventional cancer therapies, which result in relatively non-specific and often serious toxicity or impotence, more specific treatment modalities attempt to inhibit or kill malignant cells selectively while leaving healthy cells intact.

One possible treatment approach for prostate diseases is gene therapy, whereby a gene of interest is introduced into the malignant cell. Boulikas (1997) *Anticancer Res.* 17:1471-1505. The gene of interest may encode a protein which converts into a toxic substance upon treatment with another compound, or an enzyme that converts a prodrug to an active drug. For example, introduction of the herpes simplex gene encoding thymidine kinase (HSV-tk) renders cells conditionally sensitive to ganciclovir (GCV). Zijlstra et al. (1989) *Nature* 342: 435; Mansour et al. (1988) *Nature* 336: 348; Johnson et al. (1989) *Science* 245: 1234; Adair et al. (1989) *Proc. Natl. Acad. Sci. USA* 86: 4574; and Capecchi (1989) *Science* 244: 1288. Alternatively, the gene of interest may encode a compound that is directly toxic, such as diphtheria toxin (DT). For these treatments to be rendered specific to prostate cells, the gene of interest can be under control of a transcriptional regulatory element that is specifically (i.e. preferentially) increases transcription of an operably linked polynucleotide in the prostate cells. Cell- or tissue-specific expression can be achieved by using cell-specific enhancers and/or promoters. See generally, Huber et al. (1995) *Adv. Drug Delivery Rev.* 17:279-292.

A variety of viral and non-viral (e.g., liposomes) vehicles, or vectors, have been developed to transfer these genes. Of the viruses, retroviruses, herpes virus, adeno-associated virus, Sindbis virus, poxvirus and adenoviruses have been proposed for use in gene transfer, with retrovirus vectors or adenovirus vectors being the focus of much current research. Verma and Somia (1997) *Nature* 389:239-242. Adenoviruses are among the most easily produced and purified, whereas retroviruses are unstable, difficult to produce

and to purify, and may integrate into the host genome, raising the possibility of dangerous mutations. Moreover, adenovirus has the advantage of effecting high efficiency of transduction and does not require cell proliferation for efficient cell transduction. For general background references regarding adenovirus and development of adenoviral vector systems, see Graham et al. (1973) *Virology* 52:456-467; Takiff et al. (1981) *Lancet* 11:832-834; Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Graham (1984) *EMBO J* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; and Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

When used as gene transfer vehicles, adenovirus vectors are often designed to be replication-defective and are thus deliberately engineered to fail to replicate in the target cells of interest. In these vehicles, the early adenovirus gene products E1A and/or E1B are deleted and provided *in trans* by the packaging cell line 293. Graham et al. (1987) *J. Gen. Virol* 36:59-72; Graham (1977) *J. Genetic Virology* 68:937-940. The gene to be transduced is commonly inserted into adenovirus in the deleted E1A and/or E1B region of the virus genome. Bett et al. (1994). Replication-defective adenovirus vectors as vehicles for efficient transduction of genes have been described by, inter alia, Stratford-Perricaudet (1990) *Human Gene Therapy* 1:241-256; Rosenfeld (1991) *Science* 252:431-434; Wang et al. (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe et al. (1992) *Nat. Gent.* 1:372-378; Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld et al. (1992) *Cell* 68:143-155; Stratford-Perricaudet et al. (1992) *J. Clin. Invest.* 90:626-630; Le Gal Le Salle et al. (1993) *Science* 259:988-990; Mastrangeli et al. (1993) *J. Clin. Invest.* 91:225-234; Ragot et al. (1993) *Nature* 361:647-650; Hayaski et al. (1994) *J. Biol. Chem.* 269:23872-23875; and Bett et al. (1994).

The virtually exclusive focus in the development of adenoviral vectors for gene therapy is use of adenovirus merely as a vehicle for introducing the gene of interest, not as an effector in itself. Replication of adenovirus has been viewed as an undesirable result, largely due to the host immune response. In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor



suppressor genes, ribozymes, suicide genes, or genes which convert a prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle as well as the transient nature of gene expression.

There is a need for vector constructs that are capable of eliminating essentially all

- 5 cancerous cells in a minimum number of administrations before specific immunological response against the vector prevents further treatment.

A completely separate and unrelated area of research pertains to the description of tissue-specific transcriptional regulatory proteins.

#### 10 ***Rat Probasin (PB) Gene***

- The rat probasin (PB) gene encodes a nuclear and secreted protein, probasin, that is only expressed in the dorsolateral prostate. Dodd et al. (1983) *J. Biol. Chem.* 258:10731-10737; Matusik et al. (1986) *Biochem. Cell. Biol.* 64: 601-607; and Sweetland et al. (1988) *Mol. Cell. Biochem.* 84: 3-15. The dorsolateral lobes of the murine prostate are considered
- 15 the most homologous to the peripheral zone of the human prostate, where approximately 68% of human prostate cancers are thought to originate. Immunohistochemistry with polyclonal and monoclonal antibodies has shown dual cellular localization of PB within the cytoplasm and nucleus of epithelial cells of the prostate. The expression of this gene is mediated by both zinc and testosterone (T), or a derivative thereof, via the androgen
- 20 receptor (AR). T, the dominant testicular androgen, diffuses passively into the cell and either binds directly to the AR, or undergoes enzymatic reduction to 5 $\alpha$ -dihydrotestosterone (DHT), or aromatization to estrogens. Once T or DHT binds to the AR, the protein undergoes conformational changes, chaperone proteins such as heat shock proteins dissociate from the receptor, and the activated receptor can then bind DNA.
- 25 Johnson et al. (1988) in *Steroid Receptors and Disease* (Sheridan, ed.), pp. 207-228, Dekker, New York; and Chan et al. (1989) in *Pediatric Endocrinology* (Collu et al., eds.), pp. 81-124, Raven Press, New York.

- The androgen-activated AR binds to specific DNA enhancer sequences called androgen-responsive elements (AREs or ARE sites). Once anchored to an ARE, the AR is
- 30 able to regulate transcriptional activity in either a positive or negative fashion. Lindzey et al. (1994) *Vitamins and Hormones* 49: 383-432. The 5' TRE (transcriptional response

element) region of PB gene contains two ARE sites required for androgen regulation.

Rennie et al. (1993) *Mol. Endocrinol.* 7:23-36; International Application

PCT/CA93/00319, published as WO 94/03594, 17 February 1994, to Matusik.

The AR belongs to a nuclear receptor superfamily whose members are believed to  
5 function primarily as transcription factors that regulate gene activity through binding to  
specific DNA sequences, hormone-responsive elements. Carson-Jurica et al. (1990)  
*Endocr. Rev.* 11: 201-220. This family includes the other steroid hormone receptors as  
well as the thyroid hormone, the retinoic acid and the vitamin D<sub>3</sub> receptors. The  
progesterone and glucocorticoid receptor are structurally most closely related to the AR.  
10 Tilley et al. (1989) *Proc. Natl. Acad. Sci. USA* 86: 327-331; Zhou et al. (1994) *Recent*  
*Prog. Horm. Res.* 49: 249-274; and Lindzey et al. (1994) *Vit. Horm.* 49: 383-432.

Recently, the cDNAs encoding the human and rat AR have been cloned. Chang et  
al. (1988) *Proc. Natl. Acad. Sci. USA* 85: 7211-7215; Lubahn et al. (1988) *Mol.*  
*Endocrinol.* 2:1265-1275; and Trapman et al. (1988) *Biochem. Biophys. Res. Commun.*  
15 153: 241-248. The rat and human AR mRNAs show a high degree of sequence similarity  
in the coding regions and the 5' UTRs.

The AR gene itself is a target of androgenic regulation. This modulation may  
constitute an important level of control modulating physiological effects of testosterone.  
Androgen promotes up- and down-regulation of AR mRNA in a tissue- and possible stage-  
20 specific fashion. Nastiuk et al. (1994) *Endocrin.* 134: 640-649; Shan et al. (1995)  
*Endocrin.* 136: 3856-3862; and Prins et al. (1995) *Biol. Reprod.* 53: 609-619. In the testis,  
AR protein is expressed in Sertoli cells, Leydig cells and peritubular cells, but not in the  
developing germ cells. Grootegeed et al. (1977) *Mol. Cell. Endocrinol.* 9: 159-157; and  
Buzek et al. (1988) *Biol. Reprod.* 39: 39-49. Hormones such as follicle-stimulating  
25 hormone (FSH) and testosterone affect the production of AR. Verhoeven et al. (1988)  
*Endocrinology* 122: 1541-1550; and Blok et al. (1989) *Mol. Cell. Endocrinol.* 63: 267-271;  
Quarmby et al. (1990) *Mol. Endocrinol.* 4:22-28.

Up- and down-regulation of AR mRNA can be reproduced in different cell lines  
transfected with an AR cDNA. Burnstein et al. (1995); *Mol. Cell. Endocrinol.* 115:177-  
30 186 and Dai et al. (1996) *Steroids* 61:531-539. In both COS-1 and LNCaP cells  
expressing an AR cDNA, androgen promotes down-regulation of AR mRNA. Burnstein et

al. (1995). The prostate cancer cells lines PC3 and DU145 do not express an endogenous AR, but when these cells are transfected with AR cDNA, the gene demonstrates androgenic up-regulation. Dai et al. (1996). Both up- and down-regulation of AR mRNA in cells expressing the AR cDNA are due to sequences within the AR cDNA. The heterologous cytomegalovirus (CMV) promoter that drives the expression of the AR cDNA is not itself responsible for androgenic regulation of AR cDNA expression. Burnstein et al. (1995); and Dai et al. (1996). Therefore, androgen-mediated differential regulation of AR cDNA expression is conferred by the AR cDNA in a cell line-specific manner. Burnstein et al. (1995); Dai et al. (1996).

10       The molecular mechanism of AR mRNA autoregulation is complex, with both transcriptional and post-transcriptional mechanisms implicated in this process. Prins et al. (1995) *Biol. Reprod.* 53: 609-619; Wolf et al. (1993) *Mol. Endocrin.* 7: 924-936; and Blok et al. (1992) *Mol. Cell. Endocrin.* 88: 153-164. The 5' region of the AR gene does not appear to contain AREs. Blok et al. (1992) *Mol. Cell. Endocrin.* 88: 153-164. The mechanism of androgen-mediated up-regulation of AR mRNA in PC3 cells (prostate cancer cell line) expressing a transfected human AR (hAR) cDNA has been studied. An androgen-responsive region within the AR coding sequence is bound by AR and contains two distinct AREs that act synergistically to mediate AR mRNA up-regulation. Dai et al. (1996) *Mol. Endocrin.* 10:1582-1594.

20       Prostate diseases are generally recalcitrant to treatment by standard therapies. Thus, it is critical to develop new therapeutic approaches for this disease. The present invention addresses this need by providing adenoviral vectors specific for replication in AR-producing cells.

25       All publications cited herein are hereby incorporated by reference in their entirety.

#### SUMMARY OF THE INVENTION

In one embodiment, the invention provides an adenovirus vector comprising an adenovirus gene under transcriptional control of a probasin transcriptional response element (PB-TRE). The PB-TRE is capable of mediating gene expression specific to cells

which allow a PB-TRE to function, such as cells expressing the androgen receptor, e.g. prostate cells. The PB-TRE can comprise a promoter and/or enhancer from a probasin gene, provided that the PB-TRE is capable of mediating gene expression specific to cells expressing the androgen receptor. In one embodiment, a PB-TRE comprises a promoter  
5 from a probasin gene. In one embodiment, a PB-TRE comprises an enhancer from a probasin gene. In one embodiment, a PB-TRE comprises a promoter from a probasin gene and an enhancer from a probasin gene. In one embodiment, the PB-TRE is transcriptionally active in cells which allow a PB-TRE to function, such as cells expressing the androgen receptor (AR).

10 In certain embodiments, a PB-TRE comprises the nucleotide sequence of SEQ ID NO:1. In certain embodiments, a PB-TRE comprises a portion of SEQ ID NO:1 capable of mediating cell-specific transcription in AR-producing cells such as prostate cells. In another embodiment, a PB-TRE comprises the sequence from about -286 to about +28 relative to the transcriptional start site of a probasin gene (nucleotides about 141 to about  
15 454 of SEQ ID NO:1). In another embodiment, a PB-TRE comprises the sequence from about -426 to about +28 relative to the transcriptional start site of a probasin gene (nucleotide about 1 to about 454 of SEQ ID NO:1). In another embodiment, a PB-TRE comprises the sequence to about -236 to about -223 and/or the sequence to about -140 to about -117 (nucleotides about 191 to about 204 and/or about 286 to about 310,  
20 respectively, of SEQ ID NO:1), relative to the transcriptional start site of a probasin gene, combined with a probasin or non-probasin promoter. In another embodiment, a PB-TRE comprises one or two ARE sites (androgen-responsive elements) combined with a probasin or non-probasin promoter. In each embodiment, a PB-TRE is defined as a transcriptional response element or transcriptional regulatory element capable of effecting transcription in  
25 a cell, such as a prostate cell, which allows a PB-TRE to function, such as a cell expressing androgen receptor.

In certain embodiments, the adenovirus comprises a PB-TRE, which in turn comprises at least one androgen response element (ARE). In some embodiments, the ARE is ARE-1 or ARE-2 from either the probasin gene or the AR gene. In other embodiments,  
30 the adenovirus vector comprises a PB-TRE, which in turn comprises an ARE, such as

ARE-2. In other embodiments, the adenovirus vector comprises a probasin transcriptional response element, which in turn comprises both ARE-1 and ARE-2.

In some embodiments, the PB-TRE is rat in origin. In some embodiments, the rat PB-TRE is capable of mediating prostate-specific gene expression in humans.

5 In some embodiments, the adenovirus gene under control of a PB-TRE contributes to cytotoxicity (directly or indirectly), such as a gene essential for viral replication. In one embodiment, the adenovirus gene is an early gene. In another embodiment, the early gene is E1A. In another embodiment, the early gene is E1B. In yet another embodiment, both E1A and E1B are under transcriptional control of a PB-TRE. In other embodiments, the  
10 adenovirus gene essential for replication is a late gene. In various embodiments, the additional late gene is L1, L2, L3, L4, or L5. In another embodiment, the adenovirus gene under control of a PB-TRE is the adenovirus death protein gene (ADP).

In another embodiment, the adenovirus comprising an adenovirus gene under transcriptional control of a PB-TRE further comprises at least one additional adenovirus  
15 gene under transcriptional control of at least one additional prostate-specific transcriptional regulatory element. In one embodiment, a composition comprises this adenovirus. In one embodiment, this composition further comprises a pharmaceutically acceptable excipient. In one embodiment, the at least one additional prostate-specific transcriptional regulatory element is a second PB-TRE. In one embodiment, the at least one additional PB-TRE can  
20 have a sequence different from that of the first PB-TRE. In one embodiment, the at least one additional prostate-specific transcriptional regulatory element comprises a prostate-specific antigen (PSA) transcriptional regulatory element.

In other embodiments, the adenovirus vector can further comprise a heterologous gene or transgene, wherein said transgene is under transcriptional control of a PB-TRE. In  
25 one embodiment, the heterologous gene is a reporter gene. In one embodiment, the heterologous gene is conditionally required for cell survival. In some embodiments, the transgene is a cytotoxic gene.

In some embodiments, the invention provides adenovirus vector(s) complexed with a hydrophilic polymer ("masking agent") to create a masked adenovirus. The hydrophilic  
30 polymer is attached (covalently or non-covalently) to the capsid proteins of the adenovirus, particularly the hexon and fiber proteins. In preferred embodiments, the adenovirus

vectors of the instant invention are complexed with masking agents to create masked adenovirus vectors. In further preferred embodiments, the masking agent is polyethyleneglycol (PEG) covalently linked to an adenovirus vector of the instant invention.

5           In another embodiment, a method of treating prostate cancer in an individual is provided, the method comprising the step of administering to the individual an effective amount of an adenovirus vector in which an adenovirus gene is under transcriptional control of a PB-TRE. In one embodiment, the adenovirus gene is essential for viral replication. In one embodiment, the adenovirus gene is an early gene. In one embodiment, 10 the adenovirus gene is E1A. In one embodiment, the adenovirus gene is E1B. In one embodiment, the adenovirus gene is ADP. In one embodiment, the PB-TRE comprises an enhancer from a probasin gene. In one embodiment, the PB-TRE comprises a promoter from a probasin gene. In one embodiment, the PB-TRE comprises a promoter from a probasin gene and an enhancer from a probasin gene. In one embodiment, the adenovirus 15 further comprises an additional adenovirus gene under transcriptional control of at least one additional prostate-specific transcriptional regulatory element. In one embodiment, the second prostate-specific transcriptional regulatory element comprises a prostate-specific antigen (PSA) transcriptional regulatory element. In one embodiment, the additional adenovirus gene is essential for viral replication. In one embodiment, the 20 additional adenovirus gene is an early gene. In one embodiment, the additional adenovirus gene is E1A. In one embodiment, the additional adenovirus early gene is E1B. In one embodiment, the additional adenovirus gene is a late gene. In various embodiments, the late gene can be L1, L2, L3, L4, or L5. In one embodiment, the additional adenovirus gene is ADP.

25           In another aspect, the invention provides a host cell transformed with any adenovirus vector(s) described herein.

          In another aspect, the invention provides a composition comprising an adenovirus comprising an adenovirus gene under transcriptional control of a PB-TRE. In one embodiment, the composition further comprises a pharmaceutically acceptable excipient.

In another aspect, the invention provides kits which contain an adenoviral vector(s) described herein.

Another embodiment of the invention is an adenovirus which replicates preferentially in mammalian cells expressing AR.

5 In another aspect, a method is provided for propagating an adenovirus specific for cells which allow a PB-TRE to function, such as cells expressing androgen receptor, said method comprising combining any adenovirus vector(s) described herein with cells which allow a PB-TRE to function, such as cells expressing AR, whereby said adenovirus is propagated.

10 In another aspect, a method for modifying the genotype of a target cell is provided, the method comprising contacting a cell which allows a PB-TRE to function, such as a cell expressing androgen receptor, with any adenovirus described herein, wherein the adenovirus enters the cell.

In another aspect, methods are provided for detecting cells expressing probasin in a biological sample, comprising contacting cells of a biological sample with an adenovirus vector(s) described herein, and detecting replication of the adenovirus vector, if any.

In one embodiment, a method is provided for detecting cells which allow a PB-TRE to function, such as cells expressing androgen receptor in a biological sample, the method comprising the steps of: contacting a biological sample with an adenovirus vector comprising a gene under transcriptional control of a PB-TRE, under conditions suitable for PB-TRE-mediated gene expression in cells which allow a PB-TRE to function, such as cells expressing androgen receptor; and determining if PB-TRE mediates gene expression in the biological sample, where PB-TRE-mediated gene expression is indicative of the presence of cells which allow a PB-TRE to function, such as cells expressing the androgen receptor. In one embodiment, the gene is a heterologous (non-adenovirus gene). In one embodiment, the heterologous gene is a reporter gene, and production of the product of the reporter gene is detected.

25 In another embodiment, a method is provided for conferring selective toxicity on a target cell, said method comprising contacting a cell which allows a PB-TRE to function, such as a cell expressing androgen receptor, with any adenovirus disclosed herein, wherein the adenovirus enters the cell.

In one embodiment, an adenovirus is provided which comprises a heterologous gene under transcriptional control of a PB-TRE. In one embodiment, the heterologous gene is a reporter gene. In one embodiment, the heterologous gene is conditionally required for cell survival. In one embodiment, a method is provided for detecting cells  
5 which allow a PB-TRE to function, such as cells expressing androgen receptor in a sample comprising the steps of: contacting a biological sample with an adenovirus vector comprising a gene under transcriptional control of a PB-TRE, under conditions suitable for PB-TRE-mediated gene expression in cells which allow a PB-TRE to function, such as cells expressing androgen receptor; and determining if PB-TRE mediates gene expression  
10 in the biological sample, where PB-TRE-mediated gene expression is indicative of the presence of cells expressing the androgen receptor.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 depicts the sequence of the 5'-flanking region of the rat probasin (PB) gene, including the PB-TRE region.

Figures 2A and 2B depict schematic diagrams of various adenovirus vectors in which various genes are under control of a PB-TRE.

Figure 3 is a bar graph depicting results of a luciferase assay demonstrating cell-  
20 specific gene expression driven by a PB-TRE in LNCaP (PSA-plus and androgen-dependent prostate carcinoma cells) and PC-3 (PSA-minus and androgen-independent prostate carcinoma cells).

Figure 4 is a schematic depiction of an adenoviral vector in which E1A and E1B are under control of an PB-TRE, with E1A and E1B in opposite orientations.

25 Figures 5A and B are schematic depictions an adenovirus death protein (ADP) cassette for insertion into Ad. Arrows underneath Fig. 5A indicate positions of primers. Figure 5B depicts the annealed fragment containing the Y leader sequence and the ADP coding sequence.



Figure 6 is a bar graph demonstrating the reduced replication in non-prostate cells of an adenovirus, CN739, in which multiple adenoviral early genes are placed under control of prostate-specific TREs.

Figure 7 is a line graph illustrating the one-step growth curve of an adenovirus, CN739, in which multiple adenoviral early genes are placed under control of prostate-specific TREs in prostate (LNCaP) and non-prostate cells (hMVEC).

Figure 8 is a line graph showing serum PSA levels in mice treated with an adenovirus, CN739, in which multiple adenoviral early genes are placed under control of prostate-specific TREs.

Figure 9 is a line graph illustrating the efficacy in treating a prostate cancer tumor in mice of an adenovirus, CN739, in which multiple adenoviral early genes are placed under control of prostate-specific TREs.

Figure 10 is a graph depicting cytotoxicity of an adenoviral vector containing the coding sequence for adenoviral death protein (ADP), CN751 (solid squares), compared to control CN702 (solid circles), Rec 700 (solid triangles) and mock infection (Xs).

Figure 11 is a graph comparing extracellular virus yield of CN751 (solid squares) and CN702 (solid circles).

Figure 12 is a graph comparing tumor volume in mice harboring LNCaP tumor xenografts challenged with CN751 ("H"), CN702 ("J"), or buffer ("B").

Figure 13 is a schematic depiction of a method for covalent pegylation of an adenovirus. In this method, succinimidyl succinamide is used to covalently attach methoxy-PEG to adenovirus. The pegylated adenovirus is separated from the reaction components by ion exchange chromatography.

Figure 14 shows a half-tone reproduction of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (mobility shift) of pegylated adenovirus proteins. Lanes 1 and 2 are non-pegylated CN706 (control), lanes 3 through 6 are CN706 pegylated under various pH and temperature conditions (lane 3, pH 7.6, room temperature (RT); lane 4, pH 7.6, 4°C; lane 5, pH 8.2, RT; lane 6, pH 8.2, 4°C).

Figure 15 is a chromatogram of ion exchange chromatography analysis of pegylated adenovirus (PEG-706) mixed with control adenovirus CN706.

## MODES FOR CARRYING OUT THE INVENTION

We have discovered and constructed replication-competent adenovirus vectors containing a probasin (PB) transcriptional regulatory element (PB-TRE) which can preferentially replicate in cells that allow a PB-TRE to function, such as prostate cells that express the androgen receptor (AR), and have developed methods using these adenovirus vectors. The adenovirus vectors of this invention can comprise at least one adenovirus gene under the transcriptional control of a PB-TRE, which is specifically up-regulated by binding of androgen receptor. The adenovirus gene can be, for example, a gene that contributes to cytotoxicity (directly or indirectly), such as a gene that is necessary for adenoviral replication. This replication gene is preferably at least one early gene. Alternatively, the adenovirus gene under control of a PB-TRE can be an adenovirus death protein (ADP) gene. Alternatively, the adenovirus gene under control of a PB-TRE can be a late replication gene. The adenovirus can optionally comprise at least one other gene such as an adenovirus gene or transgene under control of another TRE which is different from the PB-TRE. By providing for cell-specific transcription of at least one adenovirus gene required for replication, the invention provides adenovirus vectors that can be used for specific cytotoxic effects due to selective replication. Selective replication is especially useful in the cancer context, in which targeted cell killing is desirable. The adenovirus vectors are useful for treatment of cancers such as prostate. The vectors can also be useful for detecting the presence of androgen receptor-producing cells in, for example, an appropriate biological (such as clinical) sample. Further, the adenovirus vector(s) can optionally selectively produce one or more proteins of interest in a target cell by using a PB-TRE.

We have found that the adenovirus vectors of the invention replicate preferentially in cells that allow a PB-TRE to function, such as AR-producing cells (i.e., at a significantly higher yield than in non-AR producing cells). This replication preference is indicated by comparing the level of replication (i.e., titer) in cells that allow a PB-TRE to function to the level of replication in cells that do not allow a PB-TRE to function. The replication preference is even more significant, as the adenovirus vectors of the invention

actually replicate at a significantly lower rate in cells that do not allow a PB-TRE to function than wild type virus. Comparison of the titer of a PB-producing cell type to the titer of a PB-deficient cell type provides a key indication that the overall replication preference is enhanced due to depressed replication in PB-deficient cells as well as the replication in PB-producing cells. Thus, the invention uses and takes advantage of what has been considered an undesirable aspect of adenoviral vectors, namely, their replication and possible concomitant immunogenicity. Runaway infection is prevented due to the cell-specific requirements for viral replication. Without wishing to be bound by any particular theory, the inventors note that production of adenovirus proteins can serve to activate and/or stimulate the immune system, either generally or specifically toward target cells producing adenoviral proteins which can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

Under at least some conditions, the PB-TRE is able to mediate a significantly higher degree of cell-specificity than two other prostate-specific TREs, the prostate specific antigen TRE (PSA-TRE) or the human glandular kallikrein TRE (hKLK2-TRE). In experiments with adenoviruses in which multiple adenoviral replication genes were under control of multiple prostate-specific TREs, the adenoviruses comprising PB-TRE demonstrated a significantly higher degree of cell-specific replication than viruses comprising a PSA-TRE or hKLK2-TRE. These results are described in detail in the Examples section. These findings are particularly surprising because human prostate cells were tested, and the PB-TRE is derived from rat, while the PSA-TRE and hKLK2-TRE are derived from human DNA.

As shown in the Examples section, two adenoviral vectors were constructed in which two adenovirus genes, E1A and E1B, are under control of a PSA-TRE or a PSA-TRE. E1A is arguably more important for viral replication than E1B, as E1A is expressed before any other viral genes, including E1B, and is required for E1B expression. Flint (1982) *Biochem. Biophys. Acta* 651:175-208; Flint (1986) *Advances Virus Research* 31:169-228; Grand (1987) *Biochem. J.* 241:25-38. The vector in which a PB-TRE controls E1A expression demonstrated an unexpectedly higher specificity of replication than the vector in which a PSA-TRE controls E1A. Thus, under at least some conditions, a

PB-TRE may allow a greater degree of cell-specificity of viral replication than a PSA-TRE.

The PB-TRE is also more cell-specific than the TRE of the human glandular kallikrein-1 gene (*hKLK2*) or hKLK2-TRE. Like the probasin gene, the hKLK2 gene is expressed exclusively in the prostate, is up-regulated by androgens primarily by transcriptional activation, and contains AREs in its TRE. Wolf et al. (1992) *Molec. Endocrinol.* 6:753-762. Morris (1989) *Clin. Exp. Pharm. Physiol.* 16:345-351; Qui et al. (1990) *J. Urol.* 144:1550-1556; Young et al. (1992) *Biochem.* 31:818-824; Schedlich et al. (1987) *DNA* 6:429-437; Young et al. (1992) *Biochem.* 31:818-824; Schedlich et al. (1987) *DNA* 6:429-437; and Murtha et al. (1993) *Biochem.* 32:6459-6464. As shown in the Example section, in two separate adenoviral vectors, a PSE-TRE was placed in control of E1A expression. In one vector, E1B was controlled by a PB-TRE; in the other, a hKLK2-TRE controlled E1B expression. Thus, while PB-TRE and hKLK2 are both prostate-specific, the adenoviral vector in which PB-TRE controlled E1B unexpectedly demonstrated up to 8-fold greater cell-specificity in replication than the vector in which hKLK2 controlled E1B. Thus, under at least some conditions, a PB-TRE may allow a greater degree of cell-specificity of replication in human prostate cells than either of two other prostate-specific TREs, a PSA-TRE or an hKLK2-TRE. These results are even more surprising considering that a PB-TRE is rat-derived, while both a PSA-TRE and a hKLK2-TRE are human.

Data presented in the Examples section also show that an adenovirus in which two prostate-specific TREs control replication of two adenoviral genes is capable of treating LNCaP tumors in nude mice. After palpable tumors were established, the mice were injected with adenoviruses in which a PB-TRE controls E1A, and also a PSA-TRE controls E1B. Most of the animals (4/7) were free of palpable tumors at day 42. This beneficial result was obtained with only a single dose of adenovirus. This study showed that an adenovirus of the present invention was efficacious against LNCaP tumor xenografts *in vivo*.

30

### ***General Techniques***

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Wei & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

For techniques related to adenovirus, see, inter alia, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; and Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

### ***Definitions***

A "probasin gene (PB) transcriptional response element", "probasin (PB) transcriptional regulatory element", "PB-TRE", "PBE", and the like indicate a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably-linked polynucleotide sequence in a host cell that allows a PB-TRE to function, such as a host cell that expresses androgen receptor, such as a prostate cell. A PB-TRE thus increases the level of transcription of an operably linked DNA sequence in a prostate cell relative the level of transcription in a non-prostate cell. A PB-TRE comprises at least a portion of a PB promoter and/or a PB enhancer (which may include an ARE or androgen receptor binding site). The PB-TRE can comprise a heterologous (non-PB-TRE) promoter and a PB-TRE enhancer or a PB-TRE promoter and a heterologous enhancer. Methods are described herein for measuring the activity of a PB-TRE and thus for determining whether a given cell allows a PB-TRE to function.

As described in more detail herein, a PB-TRE can comprise any number of configurations, including, but not limited to, a PB promoter; a PB enhancer; a PB promoter and a PB enhancer (preferably comprising an ARE site); a PB promoter and a non-PB (heterologous) enhancer; a non-PB (heterologous) promoter and a PB enhancer; a non-PB promoter and multiple PB enhancers; and multimers of the foregoing. Methods are described herein for measuring the activity of a PB-TRE and thus for determining whether a given cell allows a PB-TRE to function. The promoter and enhancer of a PB-TRE may be in any orientation and/or distance from the coding sequence of interest, and comprise multimers of the foregoing, as long as the desired PB cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operatively linked to) a PB-TRE. As discussed herein, a PB-TRE can be of varying lengths, and of varying sequence composition. By "transcriptional activation" or an "increase in transcription", it is intended that transcription will be increased above basal levels in the target cell (i.e. cells that allow a PB-TRE to function, such as AR-producing cell) by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, even more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably, at least about 1000-fold. Basal levels are generally the level of activity, if any, in a non-AR-producing cell, or the level of activity (if any) of a reporter construct lacking a PB-TRE as tested in an AR-producing cell. Optionally, a transcriptional terminator or transcriptional "silencer" can be placed upstream of the PB-TRE, thus preventing unwanted read-through transcription of the coding segment under transcriptional control of the PB-TRE. Also, optionally, the endogenous promoter of the coding segment to be placed under transcriptional control of the PB-TRE can be deleted.

A "functionally-preserved" variant of a PB-TRE is a PB-TRE which differs from another PB-TRE, but which still retains the ability to increase transcription of an operably linked polynucleotide, especially cell-specific transcription activity. The difference in a PB-TREs can be due to differences in linear sequence, arising from, for example, single or

multiple base mutation(s), addition(s), deletion(s), insertion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a PB-TRE.

As used herein, "prostate-specific gene expression" indicates gene expression  
5 which occurs primarily in prostate cells or in cells expressing gene products typical of prostate cells, but to a lesser degree in other cells. "Prostate-specific gene expression" indicates that this gene expression is at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, even more preferably at least  
10 about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably, at least about 1000-fold, greater in prostate cells or in cells expressing gene products typical of prostate cells than in other cells. Genes demonstrating prostate-specific gene expression include, but are not limited to, prostate specific antigen and androgen receptor.

15 A "prostate-specific transcriptional response element", "prostate-specific transcriptional regulatory element", "prostate-specific TRE", "PS-TRE" and the like indicate a DNA segment capable of mediating (i.e., regulating) and/or enhancing prostate-specific gene expression. Such a segment is typified by, but not limited to, a probasin transcriptional regulatory element (PB-TRE) and prostate-specific antigen transcriptional  
20 regulatory element (PSA-TRE), the TRE of the human glandular kallikrein-1 gene (*hGK-1* or *hKLK2*, encoding the hK2 protein), transcriptional regulatory elements thereof which are capable of mediating prostate-specific gene expression.

"Prostate-specific antigen (PSA) promoter enhancer", "Prostate-specific antigen (PSA) transcriptional regulatory element," "PSA-TRE," "prostate specific antigen  
25 promoter-enhancer," "PSE", or the like, are defined herein as the transcriptional regulatory element(s) derived from the 5' region of the prostate specific antigen gene (PSA), and sufficient to mediate prostate-specific gene expression. A PSA-TRE comprises at least a portion of a PSA-TRE promoter and/or a PSA-TRE enhancer. The PSA-TRE can comprise a heterologous (non-PSA-TRE) promoter and a PSA-TRE enhancer, or a PSA-  
30 TRE promoter and a heterologous (non-PSA-TRE) enhancer. Methods are described

herein for measuring the activity of a PSA-TRE and thus for determining whether a given cell allows a PSA-TRE to function.

The PSA-TRE depicted in SEQ ID NO:2 is the same as that given in GenBank Accession No. U37672, and published. Schuur et al. (1996) *J. Biol. Chem.* 271:7043-7051. A variant PSA-TRE nucleotide sequence is depicted in SEQ ID NO:3. This is the PSA-TRE contained within CN706 clone 35.190.13. CN706 is an adenoviral vector in which the E1A gene in Ad5 is under transcriptional control of a PSA-TRE. CN706 demonstrates selective cytotoxicity toward PSA-expressing cells *in vitro* and *in vivo*. Rodriguez et al. (1997) *Cancer Res.* 57:2559-2563. CN706 was passaged through 293 and LNCaP cells. A clone, designated 35.190.13 was isolated. The structure of this clone was confirmed by PCR, restriction endonuclease digestion and Southern blotting. Both DNA strands of the CN706 clone 35.190.13 were sequenced between positions 1 and 3537. Seven single base pair changes were found in the PSA-TRE, compared to the sequence reported by Schuur et al. (1996). These point mutations are not in the ARE and are thus not likely to affect the function of the enhancer. One mutation was found in the PSA promoter, but is not likely to affect gene expression from this promoter. In addition to these mutations, a missense mutation was found in the first exon of E1A. This C to G transition at position 3032 results in a Glu to Arg change in the E1A protein sequence. This mutation does not appear to diminish E1A function.

“Androgen receptor” as used herein refers to a protein whose function is to specifically bind to androgen and, as a consequence of the specific binding, recognize and bind to an androgen response element (ARE), following which the AR is capable of regulating transcriptional activity. The AR is a nuclear receptor that, when activated, binds to cellular androgen-responsive element(s). In normal cells the AR is activated by androgen, but in non-normal cells (including malignant cells) the AR may be activated by non-androgenic agents, including hormones other than androgens. Encompassed in the term “androgen receptor” are mutant forms of an androgen receptor, as long as the function is sufficiently preserved. Mutants include androgen receptors with amino acid additions, insertions, truncations and deletions, as long as the function is sufficiently preserved. In this context, a functional androgen receptor is one that binds both androgen and, upon androgen binding, an ARE.



An "adenovirus vector" or "adenoviral vector" (used interchangeably) is a term well understood in the art and generally comprises a polynucleotide (defined herein) comprising all or a portion of an adenovirus genome. For purposes of the present invention, an adenovirus vector contains a PB-TRE operably linked to a polynucleotide.

5 The operably linked polynucleotide can be adenoviral or heterologous. An adenoviral vector construct of the present invention can be in any of several forms, including, but not limited to, naked DNA, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex virus and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic  
10 polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically "mask" the molecule and/or increase half-life, or conjugated to a non-viral protein. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as  
15 uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus,  
20 this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted  
25 sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH<sub>2</sub>) or a mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic  
30 Acids Res.* 24: 2966-73. A phosphorothiate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995)

*Mol. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example, those described in *Current Protocols in Molecular Biology* (Ausubel et al., eds., 1987), Supp. 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania).

As used herein, "a cell which allows a PB-TRE to function", a cell in which the function of a PB-TRE is "sufficiently preserved", "a cell in which a PB-TRE is functional" is a cell in which a PB-TRE, when operably linked to, for example, a reporter gene, increases expression of the reporter gene at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably

at least about 200-fold, even more preferably at least about 400- to 500-fold, even more preferably at least about 1000-fold, when compared to the expression of the same reporter gene when not operably linked to the PB-TRE. Methods for measuring levels (whether relative or absolute) of expression are known in the art and are described herein.

5           “Under transcriptional control” is a term well-understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. As noted below, “operably linked” refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

10           As used herein, “cytotoxicity” is a term well understood in the art and refers to a state in which one or more of a cell’s usual biochemical or biological functions are aberrantly compromised (i.e., inhibited or elevated). These activities include, but are not limited to, metabolism; cellular replication; DNA replication; transcription; translation; and uptake of molecules. “Cytotoxicity” includes cell death and/or cytolysis. Assays are  
15           known in the art which indicate cytotoxicity, such as dye exclusion, <sup>3</sup>H-thymidine uptake, and plaque assays. The term “selective cytotoxicity”, as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows a PB-TRE to function when compared to the cytotoxicity conferred by the adenovirus on a cell which does not allow a PB-TRE to function. Such cytotoxicity may  
20           be measured, for example, by plaque assays, reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells or a tissue-specific marker, e.g., a cancer marker such as prostate specific antigen.

          “Replication” and “propagation” are used interchangeably and refer to the ability of  
25           a adenovirus vector of the invention to reproduce or proliferate. This term is well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. “Replication” and “propagation” include any activity directly or  
30           indirectly involved in the process of virus manufacture, including, but not limited to, viral

gene expression; production of viral proteins, nucleic acids or other components; packaging of viral components into complete viruses; and cell lysis.

A "heterologous gene" or "transgene" is any gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target  
5 cell prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

A "heterologous" promoter or enhancer is one which is not associated with or derived from a probasin gene 5' flanking sequence. Examples of a heterologous promoter or enhancer are the albumin promoter or enhancer and other viral promoters and enhancers,  
10 such as SV40.

An "endogenous" promoter, enhancer, or TRE is native to or derived from adenovirus.

The term "operably linked" relates to the orientation of polynucleotide elements in a functional relationship. A TRE is operably linked to a coding segment if the TRE  
15 promotes transcription of the coding sequence. Operably linked means that the DNA sequences being linked are generally contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable length, some polynucleotide elements may be operably  
20 linked but not contiguous.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of any vector of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate  
25 mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

A "target cell" is any cell that allows a PB-TRE to function. Preferably, a target cell is a mammalian cell which allows a PB-TRE to function, such as a cell expressing androgen receptor, preferably, a mammalian cell endogenously expressing androgen  
30 receptor, more preferably, a human cell, and more preferably, a human cell capable of allowing a PB-TRE to function and expressing an androgen receptor.

As used herein, "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer", and "cancer cells" refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. Neoplastic cells can be benign or malignant.

5 A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. The definition also includes samples that have been manipulated in any way after their  
10 procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human.  
15 Mammals include, but are not limited to, farm animals, sport animals, and pets.

An "effective amount" is an amount sufficient to effect beneficial or desired clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the  
20 disease state.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread (i.e., metastasis) of  
25 disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

A "masked adenovirus" is an adenovirus which has been complexed with a  
30 hydrophilic polymer ("masking agent"). The adenovirus may be any adenovirus, including naturally occurring isolates of adenovirus or engineered adenovirus vectors such as those

disclosed in the instant application. Masking agents are preferably of low immunogenicity. Examples of acceptable hydrophilic polymers include: polyethylene/polypropylene copolymers, polyacrylic acid analogues, sugar polymers such as cellulose, polyformaldehyde, poly(N-vinylpyrrolidone), polyethylene glycol (PEG), and the like. The hydrophilic polymer may be complexed by covalent or non-covalent attachment to the capsid proteins of the virus, particularly the hexon and fiber capsid proteins. A preferred hydrophilic polymer is PEG, and a preferred masked adenovirus is PEG covalently linked to adenovirus ("covalently pegylated adenovirus").

"Palliating" a disease means that the extent and/or undesirable clinical manifestations of a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not administering adenoviral vectors of the present invention.

*Adenoviral vectors having replication specificity for androgen receptor-producing cells*

The present invention also provides adenoviral vector constructs which comprise an adenoviral gene under transcriptional control of a PB-TRE. Preferably the adenovirus gene is one that contributes to cytotoxicity (whether directly and/or indirectly), more preferably one that contributes to or causes cell death, and even more preferably the adenoviral gene under transcriptional control of a PB-TRE is a gene essential for adenoviral replication. Examples of an adenoviral gene that contributes to cytotoxicity include, but are not limited to, an adenoviral death protein (ADP). When the adenovirus vector(s) is selectively (i.e. preferentially) replication-competent for propagation in target cells allowing a PB-TRE to function, such as cells expressing androgen receptor (AR), these cells will be preferentially killed upon adenoviral proliferation. By combining the adenovirus vector(s) with the mixture of prostate and non-prostate cells, *in vitro* or *in vivo*, the adenovirus vector(s) preferentially replicate in the target prostate cells. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic replication, the adenovirus vector replication is significantly reduced, thus lessening the probability of runaway infection and undesirable bystander effects. *In vitro* cultures may be retained to continually monitor the mixture (such as, for example, a biopsy or other appropriate

biological sample) for occurrence (i.e. presence) and/or recurrence of the target cell, e.g., any cell that allows a PB-TRE to function, such as a an androgen receptor-producing cancer cell. To ensure cytotoxicity further, one or more transgenes having a cytotoxic effect may also be present and under selective transcriptional control. In this embodiment, one may provide higher confidence that the target cells will be destroyed. Additionally, or alternatively, an adenovirus gene that contributes to cytotoxicity and/or cell death (such as ADP) may be included in the adenoviral vector, either free of, or under, selective transcriptional control.

The PB-TREs used in this invention are derived from rodent cells, including, but not limited to, rat. Preferably the PB-TRE is derived from rat cells. In one embodiment, the PB-TRE comprises a promoter of a probasin gene. In one embodiment, the PB-TRE comprises an enhancer from a probasin gene. In another embodiment, the PB-TRE comprises a promoter from a probasin gene and an enhancer from a probasin gene. In certain embodiments wherein the PB-TRE comprises an enhancer from a probasin gene, the enhancer may be in combination with a promoter from a probasin gene or a promoter from another gene. In certain embodiments wherein the PB-TRE comprises a promoter from a probasin gene, the promoter may be in combination with an enhancer from a probasin gene or an enhancer from another gene. In addition, the PB-TRE can comprise multiple promoters and/or multiple enhancers derived from the probasin gene or another gene or other genes.

A DNA fragment comprising the 5'-flanking PB DNA, nt about -426 to about +28 (SEQ ID NO:1), carries sufficient information to direct prostate-specific, developmentally- and hormonally-regulated expression of a heterologous (non-probasin) gene in transgenic mice. Greenberg et al. (1994) *Mol. Endocrinol.* 8: 230-239; Foster et al. (1997) *Cancer Res.* 57:3325-30. Furthermore, this expression was both male-specific and restricted to the epithelial cells of the lateral, dorsal, and ventral prostate lobes. The demonstration that the foreign gene activity approached precastration levels when transgenic mice were supplemented with testosterone indicates that the PB-driven reporter transgene was responding to androgens *in vivo*. Moreover, a PB-TRE could drive expression of the simian virus 40 large tumor antigen-coding region in the prostate of the transgenic mice. Greenberg et al. (1995) *Proc. Natl. Acad. Sci.* 92: 3439-3443.

Accordingly, in one embodiment, a PB-TRE is the sequence upstream of the probasin coding segment, comprising, for example, the sequence shown in Fig. 1 (SEQ ID NO:1). This sequence, e.g. from about -426 to about +28 relative to the transcriptional start site, comprises protein binding sites believed to be important or essential in cell-specific transcription, including ARE-1, ARE-2, a CAAT box, and a TATAA box.

Alternatively, a PB-TRE comprises, for example, the fragment of DNA upstream of the PB gene between base pairs about -286 and about +28 relative to the transcriptional start (nucleotides about 141 to about 454 of SEQ ID NO:1). Rennie et al. (1993) *Mol. Endocrinol.* 7:23-36. Sequence analysis revealed that this PB-TRE contains two ARE sites (designated ARE-1, also known as ARBS-1, which resembles a glucocorticoid response element, at about -236 to about -223 relative to the transcriptional start (nucleotides about 191 to about 204 of SEQ ID NO:1); and ARE-2, also known as ARBS-2, which is a unique sequence at about -140 to about -117 (nucleotides about 286 to about 310 of SEQ ID NO:1) required for androgen regulation. A single base mutation in ARE-1 or ARE-2 can result in the loss of androgen induction. Rennie et al. (1993) *Mol. Endocrinol.* 7:23-36. A fragment of 5'-flanking PB DNA containing the two ARE sites could drive expression of the bacterial chloramphenicol acetyltransferase (CAT); expression was prostate-specific and inducible by androgens, but not by glucocorticoids. Greenberg et al. (1994) *Mol. Endocrinol.* 8:230-239. Like the probasin gene, the AR gene itself is regulated by two ARE sites upstream of the coding segment. The first AR gene ARE site, ARE-1, resembles a half-site of the palindromic hormone response element and the second, ARE-2, is identical to a portion of the probasin sequence. Dai et al. (1996) *Mol. Endocrinol.* 10:1582-94. A PB enhancer is exemplified by an ARE site or pair of ARE sites, or any other sequence capable of assisting a promoter in prostate-specific transcription. Proper spacing between ARE sites may also be important in their function.

A PB-TRE can also comprise multimers. For example, a PB-TRE can comprise a tandem series of at least two, at least three, at least four, at least five PB promoter fragments. Alternatively, a PB-TRE could have one or more PB promoters along with one or more PB enhancers. These multimers may also contain non-PB promoter and/or enhancer sequences. Multiple AREs have been joined together in constructing a transcriptional regulatory element that is highly inducible by androgen receptor *in vitro*.



Snoek et al. (1996) *J. Steroid Biochem. Mol. Biol.* 59:243-50. Base substitutions between ARE sites, if multiple ARE sites are included in a single PB-TRE, as is known in the art, are unlikely to cause alterations in cell-specific transcription, although deletions may decrease or increase transcription if they bring binding sites too close or too far away or rotate them so they are on opposite sides of the DNA helix, as is known in the art. Thus, while the inventors are not wishing to be bound by a single theory, it is possible that certain modifications will result in modulated resultant expression levels, including enhanced cell-specific expression levels.

Any number of minor variations of the disclosed PB-TRE sequences are capable of mediating cell-specific transcription in cells which allow a PB-TRE to function, such as androgen receptor-producing cells. Certain point mutations within a hexamer conserved in both the ARE-1 and ARE-2 sites in the PB-TRE are known to decrease AR binding and gene activation. Rennie et al. (1993). Taking into account the ARE consensus sequence and similarity of the AREs to the glucocorticoid-responsive element (GRE) motif found in androgen regulation of the C3(1) gene, PSA gene and the sex-limited protein gene [Rennie et al. (1993); Claessens et al. (1989) *Biochem. Biophys. Res. Comm.* 164:833-840; De Vos et al. (1991) *J. Biol. Chem.* 266:3439-3443; Riegman et al. (1992) *Mol. Endocrin.* 5: 1921-1930; Adler et al. (1991) *Mol. Endocrin.* 5:1587-1596; and Dai et al. (1996) *Mol. Endocrinol.* 10:1582-94], one of skill in the art would recognize that some alterations of highly conserved bases in and around the AREs are likely to negatively affect gene activation and cell-specificity, while alterations in bases which are not highly conserved are not likely to have such effects. Certain mutations are also capable of increasing PB-TRE activity, such as the alteration within ARE-2 of four bases at -130 to -127 from CCAA to TACT or GTCT, which demonstrated greater induction than the wild-type PB-TRE. International Application PCT/CA93/00319, published as WO 94/03594, 17 February 1994, to Matusik. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in AR-expressing and non-AR-expressing cells.

As an example of how PB-TRE activity can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such

as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT),  $\beta$ -galactosidase (encoded by the *lacZ* gene), luciferase, (encoded by the *luc* gene), alkaline phosphatase, green fluorescent protein, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative PB-TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE dextran. Suitable host cells include any cell type that produces androgen receptor, including but not limited to, prostate cells, including prostate tumor cells such as LNCaP. A gene encoding androgen receptor can be transformed into and expressed in any cell that does not normally express AR; in such a cell, a PB-TRE will be functional. Non-androgen receptor producing cells, such as HLF, HLE, and 3T3 and the non-AR-producing prostate cancer cells PC3 and DU145 can be used as a control. Results are obtained by measuring the level of expression of the reporter gene using standard assays. The comparison of expression between AR-producing cells and the control cell indicates the presence or absence of transcriptional activation. Figure 3 is a bar graph depicting results of a luciferase assay demonstrating cell-specific gene expression driven by a PB-TRE in LNCaP (PSA-plus and androgen-dependent prostate carcinoma cells) and PC-3 (PSA-minus and androgen-independent prostate carcinoma cells).

A PB-TRE of the present invention may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element known in the art) can assist in shutting off transcription (and thus replication) in non-permissive (i.e., non-AR-producing) cells. Thus, presence of a silencer can confer enhanced cell-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced cell-specific replication due to more effective replication in target cells.

As is readily appreciated by one skilled in the art, a PB-TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily

available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite cell-specific transcription function.

Hence, the invention also includes adenovirus vectors comprising functionally preserved variants of the PB-TRE nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. It is possible that certain base modifications will result in enhanced expression levels or cell-specificity. Achievement of enhanced expression levels may be especially desirable in the case of more aggressive forms of prostate carcinoma, or when a more rapid and/or aggressive pattern of cell killing is warranted (due to an immunocompromised condition of the individual, for example).

Various replication-competent adenovirus vectors can be made according to the present invention in which a single or multiple adenovirus gene(s) are under control of a PB-TRE.

For example, a PB-TRE may be introduced into an adenovirus vector immediately upstream of and operably linked to (i.e. oriented in such a way as to be able to drive expression of) a replication gene, e.g. an early gene such as E1A or E1B or a late gene such as L1, L2, L3, L4, or L5.

Various other replication-competent adenovirus vectors can be made according to the present invention in which, in addition to having an adenovirus gene(s) are under control of a PB-TRE, other adenovirus gene(s) are under control of another exogenous (non-adenovirus) promoter. This promoter may be a tissue-specific promoter-enhancer, for instance the PSA-TRE (prostate-specific antigen transcriptional regulatory element) of the prostate specific antigen gene (PSA), which is preferentially expressed in prostate cells. In one embodiment, the PSA-TRE comprises an approximately 1.5 kb enhancer and a 0.5 kb promoter segment derived from the native region upstream of the PSA coding segment. The enhancer in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the prostate specific antigen (PSA) gene. The promoter consists of about nt -540 to nt about +12. In another embodiment, the PSA-TRE is present on a single fragment about 5 to about 6 kb upstream from the transcriptional start site and comprising a PSA-TRE enhancer and a PSA-TRE promoter. The enhancer contains three regions that bind prostate-specific DNA binding proteins, one of which contains a putative androgen

response element. The promoter contains typical TATA and CAAT boxes as well as a second putative androgen response element. A PSA-TRE can comprise a non-PSA-TRE promoter in combination with a PSA-TRE enhancer(s), or a PSA-TRE promoter in combination with a non-PSA-TRE enhancer(s), provided that the combination mediates prostate-specific gene expression. The PSA-TRE is more fully described in, inter alia, U.S. Patent Nos. 5,648,478 and 5,698,443; and Lundwall et al. (1987) *FEBS Lett.* 214: 317; Lundwall (1989) *Biochim. Biophys. Res. Commun.* 161:1151-1159; Riegmann et al. (1991) *Molec. Endocrin.* 5: 1921; Schuur et al. (1996) *J. Biol. Chem.* 271: 7043-7051; and Zhang et al. (1997) *Nucleic Acids Res.* 25:3143-50.

A PB-TRE may be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A, and the PSA-TRE may be introduced immediately upstream of and operably linked to another early gene such as E1B. Alternatively, a PB-TRE may be introduced upstream of and operably linked to E1B, while the PSA-TRE is introduced immediately upstream of and operably linked to E1A.

In one embodiment, E1A and E1B are under control of one or more PB-TREs by making the following construct. In wild-type adenovirus, E1A and E1B are in tandem orientation. A fragment containing the coding region of E1A through the E1B promoter is excised from the adenovirus genome and reinserted in the opposite orientation (Figure 4). In this configuration, the E1A and E1B promoters are next to each other, followed by E1A coding segment in opposite orientation (so that neither the E1A or E1B promoters are operably linked to E1A), followed by E1B in opposite orientation with respect to E1A. An PB-TRE(s) can be inserted between E1A and E1B coding regions, (which are in opposite orientation), so that these regions are under control of the TRE(s). Appropriate promoter sequences are inserted proximal to the E1A and E1B region as shown in Figure 4. Thus, an PB-TRE may drive both E1A and E1B. Such a configuration may prevent, for example, possible loop-out events that may occur if two PB-TREs were inserted in intact (native) Ad genome, one each 5' of the coding regions of E1A and E1B. By introducing a polycloning site between E1A and E1B, other types of prostate-specific TREs can be inserted, such as a transcriptional regulatory element of the prostate-specific antigen (PSA-TRE); or other cell-specific regulatory elements, preferably those associated with a disease state, such as neoplasm. Thus, this construct may find general use for cell-specific,

temporal, or other means of control of adenovirus genes E1A and E1B, thereby providing a convenient and powerful way to render adenoviral replication dependent upon a chosen transcriptional parameter.

As shown in the Examples section, adenovirus vectors in which more than one  
5 adenovirus replication gene is under control of a prostate-specific TRE demonstrated an unexpectedly higher degree of cell-specificity of replication compared to adenovirus vectors in which only one gene is controlled by a PB-TRE. In one vector, placing both E1A and E1B under transcriptional control of copies of a PB-TRE demonstrated synergy; the cell-specificity of replication was unexpectedly better than that when only E1A was  
10 controlled by PB-TRE. In another vector, placing two adenoviral replication genes under control of two different prostate-specific TREs, a PB-TRE and a PSA-TRE, allowed a much greater specificity than placing a single gene under control of a prostate-specific TRE (PSA-TRE).

Various other replication-competent adenovirus vectors can be made according to  
15 the present invention in which, in addition to having a single or multiple adenovirus gene(s) are under control of a PB-TRE, reporter gene(s) are under control of a PB-TRE.

For example, a PB-TRE may be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A or E1B, and this construct may also contain a second PB-TRE driving expression of a reporter gene. The reporter  
20 gene can encode a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT),  $\beta$ -galactosidase (encoded by the *lacZ* gene), luciferase, alkaline phosphatase, green fluorescent protein, and horse radish peroxidase. For detection of a putative prostate cell(s) in a biological sample, the biological sample may be contacted with a modified adenoviral vector in which a reporter gene (e.g., luciferase) is under  
25 control of a PB-TRE. The PB-TRE will be transcriptionally active in cells that allow a PB-TRE to function (e.g. those cells expressing androgen receptor), and luciferase will be produced. This production allows detection of cells producing androgen receptor in, for example, a human host or a biological sample. Alternatively, an adenovirus vector can be constructed in which the gene encoding a product conditionally required for survival (e.g.,  
30 an antibiotic resistance marker) is under control of a PB-TRE. When this adenovirus vector is introduced into a biological sample, cells producing androgen receptor will

become antibiotic resistant. An antibiotic can then be introduced into the medium to kill cells that do not allow a PB-TRE to function.

By "transcriptional activation" or an "increase in expression", it is intended that transcription is increased above basal levels in the target cell (i.e., a cell that allows a PB-TRE to function, such as an AR-producing cell) by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold. Comparisons between or among various PB-TREs can be assessed, for example, by measuring and comparing levels of expression within a single AR-producing cell line. It is understood that absolute transcriptional activity of a PB-TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of a PB-TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

It is understood that, to practice this invention, it is not necessary to use PB-TREs having maximum activity, or having minimum size. The requisite degree of activity is determined, inter alia, by the anticipated use and desired result. For example, if an adenoviral vector of the invention is used to monitor cells for androgen receptor-producing activity, it is possible that less than maximal degree of responsiveness by a PB-TRE will suffice to qualitatively indicate the presence of such cells. Similarly, if used for treatment or palliation of a disease state, less-than-maximal responsiveness may be sufficient for the desired result, if, for example, the androgen receptor-producing cells are not especially virulent and/or the extent of disease is relatively confined.

The size of a PB-TRE will be determined in part by the capacity of the adenoviral vector, which in turn depends upon the contemplated form of the vector (see below).

Generally a minimal size is preferred, as this provides potential room for insertion of other sequences which may be desirable, such as transgenes (discussed below) or other additional regulatory sequences. However, if no additional sequences are contemplated, or if, for example, an adenoviral vector will be maintained and delivered free of any viral packaging constraints, a larger DNA sequence may be used as long as the resultant adenoviral vector is rendered replication-competent.

If no adenovirus sequences have been deleted, an adenoviral vector can be packaged with extra sequences totaling up to about 5% of the genome size, or approximately 1.8 kb. If non-essential sequences are removed from the adenovirus genome, then an additional 4.6 kb of insert can be tolerated (i.e., a total of about 1.8 kb plus 4.6 kb, which is about 6.4 kb). Examples of non-essential adenoviral sequences that can be deleted are E3 and E4 (as long as the E4 ORF6 is maintained).

In order to minimize non-specific replication, endogenous (i.e., adenovirus) TREs should preferably be removed. This would also provide more room for inserts in an adenoviral vector, which may be of special concern if an adenoviral vector will be packaged as a virus (see below). Even more importantly, deletion of endogenous TREs would prevent a possibility of a recombination event whereby a PB-TRE is deleted and the endogenous TRE assumes transcriptional control of its respective adenovirus coding sequences (thus allowing non-specific replication). In one embodiment, an adenoviral vector of the invention is constructed such that the endogenous transcription control sequences of an adenoviral gene(s) are deleted and replaced by a PB-TRE. However, endogenous TREs may be maintained in the adenovirus vector(s), provided that sufficient cell-specific replication preference is preserved. These embodiments can be constructed by providing a PB-TRE intervening between the endogenous TRE and the replication gene coding segment. Requisite cell-specific replication preference is indicated by conducting assays that compare replication of the adenovirus vector in a cell expressing androgen receptor with replication in a non-androgen receptor producing cell. Generally, it is intended that replication is increased above basal levels in the target cell (i.e., a cell that allows a PB-TRE to function, such as a AR-producing cell) by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-

fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold. The acceptable differential can be determined empirically (using, for example, Northern assays or other assays known in the art or assays described in the Examples section) and will depend upon the

5 anticipated use of the adenoviral vector and/or the desired result.

Suitable target cells are any cell type that allows a PB-TRE to function. Preferred are cells that express or produce androgen receptor, including, but not limited to, tumor cells expressing androgen receptor. Especially preferred are prostate carcinoma cells and any metastases that produce AR. Especially preferred are those cells in which androgen  
10 receptor production can be measured using assays standard in the art such as RIA, ELISA, or Western blots (immunoassays) to determine levels of AR protein production or Northern blots to determine levels of AR mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to transcriptionally activate a PB-TRE (i.e., allow an AFP-TRE to function).

15 Any of the various serotypes of adenovirus can be used, such as Ad2, Ad5, Ad12, and Ad40. For purposes of illustration the serotype Adenovirus 5 (Ad5) is exemplified herein.

In some embodiments, a PB-TRE is used with an adenovirus gene that is essential for propagation, so that replication-competence is preferentially achievable in the target  
20 cell that allow a PB-TRE to function, such as a cell expressing androgen receptor. Preferably, the gene is an early gene, such as E1A, E1B, E2, or E4. (E3 is not essential for viral replication.) More preferably, the early gene under PB-TRE control is E1A and/or E1B. More than one early gene can be placed under control of a PB-TRE or another prostate-specific TRE. Example 1 provides a more detailed description of such constructs.

25 The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. E1A protein acts as a *trans*-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early  
30 times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* 651:175-208; Flint (1986) *Advances Virus Research* 31:169-228; Grand (1987) *Biochem. J.* 241:25-38. In the



absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced. Nevins (1989) *Adv. Virus Res.* 31:35-81. The transcription start site of Ad5 E1A is at nt 498 and the ATG start site of the E1A protein is at nt 560 in the virus genome.

5       The E1B protein functions *in trans* and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis.

10   Bailey et al. (1993) *Virology* 193:631; Bailey et al. (1994) *Virology* 202:695-706. E1B proteins are also necessary for the virus to overcome restrictions imposed on viral replication by the host cell cycle and also to reduce the apoptotic effects of E1A. Goodrum et al. (1997) *J. Virology* 71:548-561. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box.

15       The E2 region of adenovirus codes for proteins related to replication of the adenoviral genome, including the 72-kDa DNA-binding protein, the 80-kDa precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during

20   late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

      The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and

25   an ATF transcription factor binding site.

      For a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Imm.* (1995) 199 part 3:177-194.

      The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2

30   early promoter overlaps only for a few base pairs with sequences coding for a 33 kDa protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part

of the stop codon for the above mentioned 33 kDa protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a PB-TRE having SpeI ends into the SpeI site in the l-strand would disrupt the endogenous E2  
5 early promoter of Ad5 and should allow AR-restricted expression of E2 transcripts.

The E4 gene produces a number of transcription products. The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames (ORFs) 3 and 6 can both perform these functions by binding the 55-kDa protein  
10 from E1B and heterodimers of E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55-kDa protein for activity while the ORF 3 protein does not. In the absence of functional protein from ORF 3 and ORF 6, plaques are produced with an efficiency less than  $10^{-6}$  that of wild type virus. To further restrict viral replication to cells that allow a PB-TRE to function, such as AR-producing cells, E4 ORFs 1-3 can be deleted, making  
15 viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a vector with sequences in which the E1B region is regulated by a PB-TRE, a virus can be obtained in which both the E1B function and E4 function are dependent on a PB-TRE driving E1B.

The major late genes relevant to the subject invention are L1, L2, L3, L4, and L5,  
20 which encode proteins of the Ad5 virus virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. The late genes are all under the control of the major late promoter (MLP), which is located in Ad5 at about +5986 to about +6048.

In addition to conferring selective cytotoxic and/or cytolytic activity by virtue of  
25 preferential replication competence in cells that allow a PB-TRE to function, such as cells expressing androgen receptor, the adenovirus vectors of this invention can further include a heterologous gene (transgene) under the control of a PB-TRE. In this way, various genetic capabilities may be introduced into target cells allowing a PB-TRE to function, such as cells expressing androgen receptor, particularly prostate carcinoma cells. For example, in  
30 certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of

the androgen receptor-producing target cell. This could be accomplished by coupling the cell-specific replicative cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these  
5 types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin [Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507;  
10 Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415], genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins,  
15 where the pathogen proliferates intracellularly, genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. aprotinin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN- $\alpha$ , - $\beta$ , - $\gamma$ , TNF- $\alpha$ , - $\beta$ , TGF- $\alpha$ , - $\beta$ , NGF, and the like. The  
20 positive effector genes could be used in an early phase, followed by cytotoxic activity due to replication.

In some embodiments, the adenovirus death protein (ADP), encoded within the E3 region, is maintained (i.e. contained) in the adenovirus vector (Figure 5). The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is  
25 important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement. See Example 6.

Accordingly, the invention provides an adenoviral vector that includes a  
30 polynucleotide sequence encoding an ADP. A DNA sequence encoding an ADP and the amino acid sequence of an ADP are depicted in SEQ ID NO:21 and SEQ ID NO:22,

respectively. Briefly, an ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which ADP has been more fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important sequence for correct expression of late genes) is also obtained and ligated to the ADP coding sequence. The  
5 ADP coding sequence (with or without the Y leader) can then be introduced into the adenoviral genome, for example, in the E3 region (where the ADP coding sequence will be driven by the MLP or the E3 promoter). The ADP coding sequence could also be inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively, the  
10 ADP coding sequence could be operably linked to a heterologous promoter (with or without enhancer(s)), including, but not limited to, another viral promoter, a prostate-specific promoter such as the rat probasin TRE (PB-TRE).

In some embodiments, the invention provides adenoviral vectors which comprise an additional adenovirus gene under transcriptional control of a second PB-TRE. Examples of an additional adenovirus gene under transcriptional control is ADP (discussed  
15 above) and genes necessary for replication, such as early genes. For example, an adenoviral vector can be constructed such that a first PB-TRE regulates transcription of one early gene, such as E1A or E1B, and a second PB-TRE regulates transcription of another early gene. These multiple constructs may be more desirable in that they provide more than one source of cell specificity with respect to replication. As shown in the  
20 Examples section, such a double construct successfully inhibited tumor growth in mice harboring tumor xenografts.

Any of the adenoviral vectors described herein can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs. Adenoviral vectors can, alternatively, comprise polynucleotide constructs that are complexed with  
25 agents to facilitate entry into cells, such as cationic liposomes or other compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents to enhance or dampen an immune response; or complexed with agents that facilitate *in vivo* transfection, such as DOTMA, DOTAP™, and  
30 polyamines.

The invention also provides an adenovirus capable of replicating preferentially in cells which allow a PB-TRE to function, such as androgen-receptor-producing cells.

“Replicating preferentially in an androgen-receptor-producing cell” means that the adenovirus replicates more in a cell producing all the factors and co-factors needed for PB  
5 expression than in a cell not producing such factors and co-factors. Preferably, the adenovirus replicates at a significantly higher level in cells that allow a PB-TRE to function, such as AR-producing cells than non-AR-producing cells; preferably, at least about 2-fold higher, preferably at least about 5-fold higher, more preferably at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at  
10 least about 100-fold higher, still more preferably at least about 400-to about 500-fold higher, still more preferably at least about 1000-fold higher, most preferably at least about  $1 \times 10^6$  higher. Most preferably, the adenovirus replicates solely in AR-producing cells (that is, does not replicate or replicates at very low levels in non AR-producing cells).

If an adenoviral vector comprising an adenovirus polynucleotide is packaged into a  
15 whole adenovirus (including the capsid), the adenovirus itself may be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Viol.* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus (or subgenera) could be  
20 used to further increase cell-specificity of cytotoxicity and/or cytolysis.

In some embodiments, an adenovirus vector(s) is complexed to a hydrophilic polymer to create a masked adenovirus. The hydrophilic polymer is attached (covalently or non-covalently) to the capsid proteins of the adenovirus, particularly the hexon and fiber proteins. In preferred embodiments, the adenovirus vectors of the instant invention a  
25 complexed with masking agents to create masked adenovirus vectors. Masked adenoviruses are advantageous due to (a) the masking of the adenovirus surface to adenovirus neutralizing antibodies or opsinins which are in circulation, and (b) increasing systemic circulation time of adenovirus particles by reduction of non-specific clearance mechanism in the body (i.e., macrophages, etc.). In the *in vivo* context, the systemic  
30 delivery of a masked adenovirus results in a longer circulation of viral particles, less immunogenicity, and increased biodistribution with a decrease in clearance by the liver

and spleen. Extensive research has been done on modification of proteins and lipids with hydrophilic polymers (especially PEG), but the inventors are unaware of any other use of masking agents in conjunction with adenovirus or adenovirus constructs.

Accordingly, the invention provides an adenovirus complexed with a masking agent. Preferably, the masking agent is PEG. A schematic of one method of making a masked adenovirus is depicted in Figure 13 (see also Example 8). A preferred embodiment is a masked adenovirus comprising an adenovirus vector(s) described herein, with a more preferred embodiment comprising a pegylated adenovirus comprising an adenovirus described herein. The invention also provides methods to make and use these masked adenoviruses, which are evident from the description herein.

The masking agent may be of various molecular weights, as long as the desired complex and requisite functionality is obtained. Most masking agents obtained from commercial sources are normally polydisperse in relation to the stated molecular weight (*i.e.*, the masking agent is supplied in a distribution of molecular weights surrounding the nominal molecular weight). Masking agents useful in masking adenoviruses according to the instant invention may have nominal weights of about 2000 to about 50,000; preferably, about 2500 to about 30,000; preferably, about 3000 to about 25,000; more preferably, about 5000 to about 20,000. Preferably, the nominal molecular weight is less than about 20,000, more preferably less than about 10,000, more preferably less than about 7500, more preferably less than about 5000. Preferably, the masking agent is PEG with a nominal molecular weight of less than about 5000 Da. Mixtures of different weight masking agents are also contemplated.

The masking may be covalently or non-covalently attached. In the case of non-covalent attachment, the attachment may be via electrostatic, hydrophobic, or affinity interactions. The masking agents used for non-covalent attachment may be modified masking agents (*i.e.*, the masking agent is synthesized or modified to contain particular chemical moieties not normally found in the masking agent). Masking agents useful for electrostatic attachment to adenoviral vectors will be masking agents which contain, or have been modified or synthesized to contain, charged moieties which bind to the adenovirus surface by electrostatic interaction. Negatively charged masking agents include masking agents which contain phosphate groups, sulfate groups, carboxy groups, and the

like. Quaternary amine groups are useful as positively charged moieties for electrostatic, non-covalent attachment of masking agents to adenovirus. Masking agents containing or modified or synthesized to contain hydrophobic groups, such as lipids (*e.g.*, phosphatidylethanolamine and the like) and other hydrophobic groups (such as phenyl groups or long alkyl chains), can be complexed to adenoviral vectors by hydrophobic interaction with stable hydrophobic regions on the virus. Affinity masking agents can be made using any small molecule, peptide or protein which binds to adenovirus. The affinity and hydrophobic moieties may be attached to the masking agent by any method known in the art, preferably by chemical crosslinking with a chemical crosslinker.

If the masking agent is covalently attached, a chemical crosslinker is preferably used to covalently bond the masking agent to the adenovirus. The crosslinker may be any crosslinker capable of creating a covalent linkage or bridge between the masking agent and the adenovirus. Direct crosslinking, in which the adenovirus, masking agent and a separate crosslinker molecule are reacted, may be employed to create covalently masked adenovirus, using any chemical crosslinker known in the art which will create crosslinks between the masking agent and protein. Either the masking agent or the adenovirus may be modified prior to the crosslinking reaction, so that the chemical crosslinker will react with the two molecules (*e.g.*, the masking agent may be modified to add amine groups, allowing it to be crosslinked to the adenovirus by crosslinking agents which react with amines).

Preferably, either the masking agent or the adenovirus is first activated by reaction with a crosslinking agent. Unreacted crosslinker is then removed from the masking agent or adenovirus. The activation reaction preferably results in one or two molecules of crosslinking agent per molecule of masking agent, more preferably a single molecule of crosslinking agent per molecule of masking agent. The activated masking agent or adenovirus is then mixed with adenovirus (if the masking agent is activated) or masking agent (if the adenovirus is activated) under the appropriate reaction conditions to form masked adenovirus. Preferably, the masking agent is activated, then reacted with adenovirus.

The preferred masking agent is PEG. Preferred activated PEGs include, but are not limited to: nucleophilic crosslinking PEGs such as end terminal amine PEG, PEG amino

acid esters, PEG hydrazine hydrochloride, thiol PEGs, and the like; carboxyl PEGs including succinate PEG, carboxymethylated PEG, PEG-propionic acid, and PEG amino acids; sulfhydryl-selective PEGs such as PEG-maleimide, PEG-orthopyridyl-disulfide and the like; heterofunctional PEGs including amines and acids PEG, NHS-maleimide PEG and NHS-vinylsulfone PEG; PEG silanes; biotin PEGs; Vinyl derivatives of PEG such as allyl PEG, PEG acrylate, PEG methacrylate, and the like; and electrophilic active PEGs, including PEG succinimidyl succinate, PEG succinimidyl succinamide, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, PEG2-NHS, succinimidyl esters of amino acid PEGs, pendant modified PEG NHS esters (such as those available from Innophase, Inc.), PEG-glycidyl ether (epoxide), PEG-oxycarbonylimidazole, PEG nitrophenyl carbonates, PEG trichlorophenyl carbonates, PEG treslate, PEG-aldehyde, PEG-isocyanate, copolymers of PEG allyl ether and maleic anhydride, PEG vinylsulfone, and other activated PEGs as will be apparent to one of skill in the art. The activated PEG is preferably PEG-N-hydroxysuccinimidyl succinamide or PEG-succinimidyl succinate, more preferably PEG-N-hydroxysuccinimidyl succinamide.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art, such as calcium phosphate precipitation, electroporation, direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used in packaged adenoviruses, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about  $10^4$  to about  $10^{14}$ . The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01  $\mu\text{g}$  to 1000  $\mu\text{g}$  of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an



amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Host systems are known in the art and need not be described in detail herein. Prokaryotic host cells include bacterial cells, for example, *E. coli*, *B. subtilis*, and mycobacteria.

Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Examples of fungi (including yeast) host cells are *S. cerevisiae*, *Kluyveromyces lactis* (*K. lactis*), species of *Candida* including *C. albicans* and *C. glabrata*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* (*S. pombe*), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are COS cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells. *Xenopus laevis* oocytes, or other cells of amphibian origin, may also be used. Suitable host cells also include any cells which allow a PB-TRE to function, such as cells that produce androgen receptor and/or proteins and other factors necessary for expression of the probasin gene, whether the AR and/or other factors are produced naturally or recombinantly.

The present invention also includes compositions, including pharmaceutical compositions, containing the adenoviral vectors described herein. Such compositions are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Preferably, these compositions further comprise a pharmaceutically acceptable excipient. These compositions, which can comprise an effective amount of an adenoviral vector of this invention in a pharmaceutically acceptable excipient, are suitable for systemic administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack

Publishing (1990). Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

5 The present invention also encompasses kits containing an adenoviral vector of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow someone to detect the presence of cells that allow a PB-TRE to function, such as androgen receptor-producing cells in a suitable biological sample, such as  
10 biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

15

#### ***Preparation of the adenovirus vectors of the invention***

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, a PB-TRE is inserted 5' to the adenoviral gene of interest, preferably an adenoviral replication gene, more preferably one  
20 or more early replication genes (although late gene(s) can be used). A PB-TRE can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for a PB-TRE. Accordingly, convenient restriction  
25 sites for annealing (i.e., inserting) a PB-TRE can be engineered onto the 5' and 3' ends of a PB-TRE using standard recombinant methods, such as PCR.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art such as chemical synthesis recombinant methods and/or obtained from biological sources.

30 Adenoviral vectors are conveniently prepared by homologous recombination or *in vitro* ligation of two plasmids, one providing the left-hand portion of adenovirus and the

other providing the right-hand portion, one or both of which contains at least one adenovirus gene under control of a PB-TRE. If homologous recombination is used, the two plasmids should share at least about 500 bp of sequence overlap. Each plasmid, as desired, may be independently manipulated, followed by cotransfection in a competent

5 host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from a PB-TRE for propagation of the adenovirus. Plasmids are generally introduced into a suitable host cell such as 293 cells or LNCaP cells, etc., using appropriate means of transduction, such as cationic liposomes.

Alternatively, *in vitro* ligation of the right and left-hand portions of the adenovirus genome

10 can be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner *et al.* (1983) *Nucleic Acid Research* 11:6003-6020; Bridge *et al.* (1989) *J. Virol.* 63:631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type

15 left-hand end of Ad5, from Adenovirus 5 nt 22 to 5790. pBHG10 (Bett. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806; Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The deletion in E3 provides room in the virus to insert a 3-kb PB-TRE without deleting the endogenous enhancer-promoter. The gene for E3 is located on the opposite strand from E4 (r-strand). pBHG11 [Bett. *et al.*

20 (1994)] provides an even larger E3 deletion (an additional 0.3 kb is deleted).

For manipulation of the early genes, the transcription start site of Ad5 E1A is at nt 498 and the ATG start site of the E1A coding segment is at nt 560 in the virus genome. This region can be used for insertion of a PB-TRE. A restriction site may be introduced by employing polymerase chain reaction (PCR), where the primer that is employed may be

25 limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a 30 sequence change resulting in a unique restriction site, one can provide for insertion of PB-TRE at

30 that site. Example 1 provides a more detailed description of an adenoviral vector in which E1A is under PB-TRE control.

A similar strategy may be used for insertion of a PB-TRE to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from Ad5 nt 1636 to 1701. By insertion of a PB-TRE in this region, one can provide for cell-specific transcription of the E1B gene. By employing the left-hand region modified with the cell-specific response element regulating E1A, as the template for introducing a PB-TRE to regulate E1B, the resulting adenovirus vector will be dependent upon the cell-specific transcription factors for expression of both E1A and E1B. Example 1 provides a more detailed description of how such constructs can be prepared.

10 Similarly, a PB-TRE may be inserted upstream of the E2 gene to make its expression cell-specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Imm.* (1995) 199 part 3:177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable to genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33-kDa protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kDa protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a PB-TRE having SpeI ends into the SpeI site in the l-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow AR-restricted expression of E2 transcripts.

25 For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at about nt 35609, the TATA box at about nt 35638 and the first AUG/CUG of ORF1 is at about nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a PB-TRE may be introduced upstream from the transcription start site. For the construction of a full-length adenovirus with a PB-TRE inserted in the E4 region, the co-transfection and homologous

recombination are performed in W162 cells (Weinberg *et al.* (1983) *Proc. Natl. Acad. Sci.* 80:5383-5386) which provide E4 proteins *in trans* to complement defects in synthesis of these proteins.

5 Methods of packaging adenovirus polynucleotides into adenovirus particles are known in the art and are described in the Examples.

The methods of preparation of masked adenovirus vary according to masking agent and the mode of attachment (covalent or non-covalent). Masked adenovirus with non-covalently attached masking agent is prepared by thoroughly and intimately mixing the adenovirus and the masking agent. Covalent crosslinking is achieved by mixing the  
10 reaction components under conditions appropriate to the crosslinking reagent. Chemical crosslinking protocols are well known in the art. The exact reaction conditions for any given chemical crosslinker will vary according to the chemistry of the crosslinker and the modifications (if any) to the PEG and the adenovirus, as will be apparent to one of skill in the art. When the masking agent is PEG, the molar ratio of adenovirus to PEG is  
15 preferably between  $1:1 \times 10^6$  and  $1:1 \times 10^7$ , more preferably about  $1:4 \times 10^6$ . For the preferred activated PEG, PEG-NHS-succinamide, the activated PEG is preferably between 0.5 to 5 mM in the crosslinking reaction, more preferably about 2 mM. Preferably adenovirus in the crosslinking reaction is between  $10^6$  and  $10^{12}$ , more preferably about  $5 \times 10^9$ . Using the preferred activated PEG, the pH of the crosslinking reaction is preferably  
20 between about 7 and 9, more preferably between about 7.5 and 8, and the reaction is preferably run for 10 to 30 minutes at a temperature ranging from about 4° C to room temperature (about 20° C).

Following the crosslinking reaction, the masked adenovirus is separated from the reaction components. The separation may be accomplished by any method known to one  
25 of skill in the art, including chromatographic methods such as size exclusion chromatography, ion exchange chromatography or hydrophobic interaction chromatography, electrophoretic methods, or filtration methods such as dialysis, diafiltration or ultrafiltration.

30

***Methods using the adenovirus vectors of the invention***

The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

In one embodiment, methods are provided for conferring selective cytotoxicity in  
5 cells which allow a PB-TRE to function, such as cells expressing androgen receptor comprising contacting the cells with an adenovirus vector described herein. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, <sup>3</sup>H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for propagating an adenovirus  
10 specific for cells that allow a PB-TRE to function, such as those cells expressing androgen receptor. These methods entail combining an adenovirus vector with cells whereby said adenovirus is propagated.

Another embodiment provides methods of killing cells that allow a PB-TRE to function, such as cells expressing the androgen receptor in a mixture of cells, comprising  
15 combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally a mixture of normal cells and cancerous cells producing androgen receptor, and can be an *in vivo* mixture or *in vitro* mixture.

The invention also includes methods for detecting cells which allow a PB-TRE to function, such as cells expressing androgen receptor in a biological sample. These  
20 methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. In one method, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. Alternatively, the sample can be contacted with an adenovirus in which a reporter gene is under control of a PB-TRE. Expression of  
25 the reporter gene indicates the presence of cells that allow the PB-TRE to function, such as androgen receptor-producing cells. Alternatively, an adenovirus can be constructed in which a gene conditionally required for cell survival is placed under control of a PB-TRE. This gene may encode, for example, antibiotic resistance. The adenovirus is introduced into the biological sample, and later the sample is treated with an antibiotic. The presence  
30 of surviving cells expressing antibiotic resistance indicates the presence of cells that allow a PB-TRE to function. A suitable biological sample is one in which androgen receptor-

producing cells may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells producing androgen receptor, such as prostate cancer cells, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to  
5 promote assay conditions such as selective enrichment and/or solubilization. In these methods, androgen receptor-producing cells can be detected using *in vitro* assays that detect proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yields) and plaque assays (which measure infectious particles per cell). Also, propagation can be detected by  
10 measuring specific adenoviral DNA replication, which are also standard assays.

The invention also provides methods of modifying the genotype of a target cell, comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention also provides methods of modifying the genotype of a target cell,  
15 comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, preferably a tumor cell that expresses androgen receptor, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the  
20 tumor cell and exhibits selective cytotoxicity for the tumor cell. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a <sup>3</sup>H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor  
25 shrinkage. "Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein.

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the  
30 present invention, wherein the adenoviral vector is selectively cytotoxic toward cells producing the tumor cell marker. Tumor cell markers include, but are not limited to, PSA,

hK2, and carcinoembryonic antigen. Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to, immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. Treatment using an adenoviral vector(s) is indicated in individuals with prostate-associated diseases as described above, such as hyperplasia and cancer. Also indicated are individuals who are considered to be at risk for developing prostate-associated diseases, such as those who have had disease which has been resected and those who have had a family history of prostate-associated diseases. Determination of suitability of administering adenoviral vector(s) of the invention will depend, inter alia, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) in a pharmaceutically acceptable excipient is administered. Pharmaceutical compositions are described above.

The amount of adenoviral vector(s) to be administered will depend on several factors, such as route of administration, the condition of the individual, the degree of aggressiveness of the disease, the particular PB-TRE employed, and the particular vector construct (i.e., which adenovirus gene(s) is under PB-TRE control).

If administered as a packaged adenovirus, from about  $10^4$  to about  $10^{14}$ , preferably from about  $10^4$  to about  $10^{12}$ , more preferably from about  $10^4$  to about  $10^{10}$ . If administered as a polynucleotide construct (i.e., not packaged as a virus), about 0.01  $\mu\text{g}$  to about 100  $\mu\text{g}$  can be administered, preferably 0.1  $\mu\text{g}$  to about 500  $\mu\text{g}$ , more preferably about 0.5  $\mu\text{g}$  to about 200  $\mu\text{g}$ . More than one adenoviral vector can be administered, either simultaneously or sequentially. Administrations are typically given periodically, while monitoring any response. Administration can be given, for example, intratumorally, intravenously or intraperitoneally.

The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective.



The following examples are provided to illustrate but not limit the invention.

## EXAMPLES

### Example 1

#### Adenovirus vectors containing an early gene under control of a transcriptional element derived from a probasin transcriptional response element (PB-TRE)

##### 1. A. The probasin transcriptional response element (PB-TRE)

The 454 nucleotide fragment (nt about -426 to about +28) of the rat PB-TRE, which contains two androgen response elements (ARE sites), a CAAT box and a TATAA box (Figure 1, SEQ ID NO:1), was amplified by polymerase chain reaction (PCR) using rat genomic DNA as template and the synthetic oligonucleotides:

42.2.1 (SEQ ID NO:4):

5'-GATCACCGGTAAGCTTCCACAAGTGCATTTAGCC-3',

PinAI site underlined,

and

42.2.2 (SEQ ID NO:5):

5'-GATCACCGGTCTGTAGGTATCTGGACCTCACTG-3',

20

or oligonucleotides

42.2.3 (SEQ ID NO:6):

5'-GATCCCGGCCGAAGCTTCCACAAGTGCATTTAGCC-3',

EagI site underlined,

25

and

42.2.4 (SEQ ID NO:7):

5'-GATCCCGGCCGCTGTAGGTATCTGGACCTCACTG-3'.

The oligonucleotides created a unique PinAI (Agi) site (A/CCGGT) or EagI site (C/GGCCG) at both ends of the PCR fragments. The PCR fragments were ligated into the pGEM-T vector (Promega) to generate plasmids CN249 and CN250. Similarly, CN256

was created using the same strategy but the PB-TRE fragment was ligated into the pCRT vector (Invitrogen); CN271 is identical to CN250 but with a HindIII site at the 5'-end. These plasmids provide the PB-TRE DNA fragments for the constructs reported below. In some of the adenovirus vectors described below, the endogenous (adenoviral) TREs were not deleted; rather, in each construct, the PB-TRE was inserted between the endogenous TRE (e.g., the E1A TRE) and its respective coding segment (e.g., the E1A coding segment). In other vectors, the endogenous (Ad5) promoter-enhancer has been deleted, and the prostate-specific promoter-enhancer placed immediately upstream of an early gene.

10

**1. B. Construction of PB-TRE adenovirus comprising an adenovirus gene under control of a prostate-specific transcriptional regulatory element**

**1. B. 1. PB-TRE-driven E1A Ad5 plasmid (CN251)**

15 An adenovirus vector in which expression of an early gene, E1A, is under control of PB-TRE was constructed as follows.

CN124 is a derivative of construct pXC.1, which contains the wild-type left-hand end of Ad5, from nt 22 to 5790, including both E1A and E1B (McKinnon (1982) *Gene* 19:33-42). CN124 also has, among other alterations, an artificial PinAI site at Ad5 nt 547 (between the E1A transcriptional start at nt 498 and the E1A coding segment beginning with ATG at 560).

20

To construct CN124 from pXC.1, we introduced an AgeI site 12 bp 5' to the E1A initiation codon (Ad5 547) by oligonucleotide-directed mutagenesis and linked PCR. To achieve this, pXC.1 was PCR-amplified using primers:

25 5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:8), containing an EcoRI site, and

5'-TTTCAGTCACCGGTGTCGGA (15.134B) (SEQ ID NO:9),  
containing an extra A to introduce an AgeI site. This created a segment from the EcoRI site in the pBR322 backbone to Ad5 560. A second segment of pXC.1 from Ad 541 to the XbaI site at Ad nucleotide 1339 was amplified using primers:

30

5'-GCATTCTCTAGACACAGGTG (15.133B) (SEQ ID NO:10) containing an XbaI site, and

5'-TCCGACACCGGTGACTGAAA (15.134A) (SEQ ID NO:11), containing an extra T to introduce an AgeI site. These two PCR-amplified DNA segments were mixed  
5 and amplified with primers 15.133A and 15.133B to create a DNA segment from the EcoRI site to the XbaI site of pXC.1. This DNA segment encompasses the leftmost 1317 bases of Ad sequence and contains an AgeI site at Ad 547. This DNA segment was used to replace the corresponding segment of pXC.1 to create CN95.

An EagI site was created upstream of the E1B start site by inserting a G residue at  
10 Ad5 1682 by oligonucleotide directed mutagenesis as above. To simplify insertion of an PB-TRE in the EagI site, the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and re-ligation to construct CN114. The primers:

5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:8), containing an EcoRI  
15 site, and

5'-GCCCACGGCCGCATTATATAC (9.4) (SEQ ID NO:12), containing an EagI site, and

5'-GTATATAATGCGGCCGTGGGC (9.3) (SEQ ID NO:13) containing an extra G and an EagI site, and

20 5'-CCAGAAAATCCAGCAGGTACC (24.020) (SEQ ID NO:14), containing a KpnI site, were used to amplify the segment between 1682 and the KpnI site at Ad5 2048. Co-amplification of the two segments with primers 15.133A and 24.020 yielded a fragment with an EagI site at Ad5 1682 which was used to replace the corresponding EcoRI/KpnI site in pXC.1 to construct CN124.

25 CN124 was linearized with PinAI and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). CN249 was digested with PinAI to free the PB-TRE fragment. The PB-TRE fragment was then ligated into the PinAI-linearized CN124, producing CN251. CN253 is similar to CN251 except for the PB-TRE fragment is in the reverse orientation.

Thus, construct CN251 contains the PB-TRE inserted upstream of and operably linked to the E1A coding segment in the Adenovirus 5 genome. The vector CN253 is similar, but the PB-TRE is in the reverse orientation.

5    **1. B. 2. PB-TRE-driven E1B Ad5 plasmid (CN254)**

An adenovirus derivative in which the expression of another early gene, E1B, is under control of the PB-TRE was constructed as follows.

CN124, which carries the left-end of Ad5, as described above, also contains an artificial *EagI* site at Ad5 nt 1682, or just upstream of the E1B coding segment. The PB-TRE fragment was excised from CN250 with *EagI* and inserted into CN124 digested with *EagI*. This produced CN254, which contains the PB-TRE immediately upstream of and operably linked to the E1B coding segment.

CN255 is identical to CN254, but the orientation of the PB-TRE insert is reversed.

CN275 is the same as CN254, but with a *HindIII* site at the 5'-end.

15

**1. C. Construction of adenovirus vectors in which expression of one adenovirus replication gene is controlled by PB-TRE, and expression of another is controlled by another prostate-specific TRE**

20    **1. C. 1. Adenovirus vector comprising PB-TRE-driven E1A and E1B driven by another promoter-enhancer, PSA-TRE (CN257)**

An adenovirus vector in which expression of the E1A gene is under control of the PB-TRE and expression of the E1B gene is under control of the prostate specific antigen transcriptional regulatory element (PSA-TRE) was constructed as follows. The PSA-TRE region has been described in detail in, inter alia, U.S. Patent Nos. 5,648,478 and 5,698,443; Lundwall (1989) *Biochim. Biophys. Res. Commun.* 161:1151-1159; and Zhang et al. (1997) *Nucleic Acids Res.* 25:3143-50.

CN125 is an (left-end) adenovirus derivative in which expression of the E1B gene is driven by a PSA-TRE, and in which an *PinAI* site lies upstream of the E1A gene, whose expression is driven by its wild-type (endogenous) promoter. CN125 was derived by

30

inserting the PSA-TRE as an *EagI* fragment from CN105 into the *EagI* site of CN124 (immediately upstream of the E1B gene).

The *PinAI* PB-TRE fragment was then inserted into CN125 digested with *PinAI*, which cleaves just upstream of E1A. This created construct CN257, which is a plasmid containing PB-TRE-driven E1A and PSA-TRE-driven E1B. CN258 is similar to CN257, but with the opposite orientation of the PB-TRE fragment.

#### **1. C. 2. Ad5 plasmid comprising PSA-TRE-driven E1A and PB-TRE-driven E1B (CN273)**

10 An adenovirus vector was constructed in which expression of E1A is mediated by the exogenous non-PSA-TRE promoter-enhancer, and expression of E1B is mediated by PB-TRE.

The construct CN257, which is a plasmid containing PB-TRE-driven E1A and PSA-TRE-driven E1B, was described above.

15 CN273 was constructed with a position change between PSA-TRE and PB-TRE in CN257. CN143 is a pBluescript (Stratagene, La Jolla, California) derivative containing the PSA-TRE fragment. This fragment was excised with *PinAI* and ligated into *PinAI*-digested CN254. The final construct is a plasmid containing PSA-TRE-driven E1A and PB-TRE-driven E1B. CN274 is similar to CN273 except for the opposite orientation of PB-TRE.

#### **1. D. Construction of adenovirus vectors in which expression of more than one adenovirus replication gene is controlled by a PB-TRE**

##### **25 1. D. 1. PB-TRE-driven E1A and PB-TRE-driven E1B Ad5 plasmid (CN268)**

An adenovirus vector in which expression of both E1A and E1B are driven by PB-TRE was constructed as follows.

CN251, described above, comprises a PB-TRE fragment inserted just upstream of the E1A coding segment.

30 CN268 was generated by inserting a second PB-TRE in front of the E1B gene in CN251. A PB-TRE fragment was excised from CN250 by *EagI*-digestion and ligated into

EagI-digested CN251 to create CN268. The final construct is a plasmid with PB-TRE driving E1A and a second PB-TRE driving E1B. CN269 is the same as CN268 but the orientation of the second PB-TRE is reversed.

In addition, in experiments (described below) in which adenoviral vectors were constructed in which two genes were under control of two separate TREs specific for prostate cells, the effect was synergistic (i.e. the increase in cell-specificity of transcription is more than additive).

## 1. E. Construction of adenovirus vectors in which expression of reporter genes is under control of a prostate-specific TRE

### 1. E. 1. PB-TRE-driven reporter gene plasmid (CN280, CN281)

An adenovirus vector containing the entire structural gene of firefly luciferase (*luc*) driven by a PB-TRE was constructed as follows.

CN280 was derived from pGL3-Basic (Promega) for transfection experiments. A PB-TRE fragment was excised from CN249 by NcoI/SacI digestion and ligated into NcoI/SacI-digested pGL3-Basic to produce CN280. The final construct is a plasmid containing the entire structural gene of *luc* driven by PB-TRE.

CN281 was generated from the same procedure except that a PB-TRE fragment was ligated into pGL3-Enhancer (Promega).

### 1. E. 2. AR up-regulation of PB-TRE-driven reporter gene expression

To test the specificity of PB-TRE-driven gene expression, the region of the PB 5'-flanking DNA (about -426 to about +28) including the endogenous promoter sequences was inserted upstream of the firefly luciferase gene to generate a chimeric PB-TRE-*luc* plasmid (CN280) (see above). Transient transfection of LNCaP (PSA-producing and AR-producing prostate carcinoma cells) and PC-3 (PSA-deficient and AR-deficient prostate carcinoma cells) cells was performed. Transfection involved the cationic lipid-mediated method under conditions standardized for another vector, pCMV- $\beta$ gal. The results presented in Figure 3 show that LNCaP cells transfected with CN280 have approximately

400 times more activity than background, indicating that the PB-TRE is intact. Further, the overall luciferase activity recovered in the cellular extracts of LNCaP cells was much higher (about 30-40-fold) than that measured in PC-3 cells. Thus, the results indicate that PB-TRE expression is up-regulated in AR-producing, PSA-producing cells, and that PB-TRE is capable of mediating specific expression in cells producing the androgen receptor.

#### **1. F. Homologous recombination for generation of the recombinant adenovirus vectors containing a PB-TRE**

Of the plasmids described above, in which an adenovirus gene is under control of a prostate-specific TRE, many contained only the left-end of adenovirus. In order to construct whole adenovirus comprising adenovirus gene(s) under control of prostate-specific TREs, these plasmids were homologously recombined with right-end adenovirus plasmids.

A human embryonic kidney cell line, 293, efficiently expresses the Ad5 E1A and E1B genes and exhibits a high transfection efficiency with adenovirus DNA. For these experiments, 293 cells were co-transfected with one left-end Ad5 plasmid and one right-end Ad5 plasmid. Homologous recombination generates whole PB-TRE-containing adenoviruses with the required genetic elements for replication in 293 cells. Briefly, 5 µg of CN251 (PB-TRE - E1A) and BHG11 (which contains wt right part of adenovirus) were co-transfected into 293 cells. The cells were overlaid with medium, and infectious virus, generated by *in vivo* recombination, was detected by cytopathic effect and isolated. Plaque-purified stocks of a vector comprising a PB-TRE, designated CN737, were established. The structure of the recombinant virus was characterized by PCR, restriction endonuclease digestion and Southern blot analysis. The viral genome of CN737 is the full-length Ad5 wherein E1A is under a control of PB-TRE.

The plasmids to be combined were co-transfected into 293 cells using cationic liposomes such as Lipofectin (DOTMA:DOPE™, Life Technologies). The two plasmids (10 µg of each in 500 µl of minimum essential medium (MEM) without serum or other additives) were mixed with a four-fold molar excess of liposomes in 200 µl of the same buffer. The DNA-lipid complexes were then placed on the cells and incubated at 37°C, 5% CO<sub>2</sub> for 16 hours. After incubation the medium was changed to MEM with 10% fetal



bovine serum and the cells are further incubated at 37°C, 5% CO<sub>2</sub>, for 10 days with two changes of medium. At the end of this time the cells and medium were transferred to tubes, freeze-thawed three times, and the lysate was used to infect 293 cells at the proper dilution to detect individual viruses as plaques.

5           Plaques obtained were plaque-purified twice, and viruses were characterized for presence of desired sequences by PCR and occasionally by DNA sequencing. For further experimentation, the viruses were purified on a large scale by cesium chloride gradient centrifugation.

          Additional viruses containing the entire adenovirus genome with one or more early  
10       genes under control of a prostate-specific TRE were also constructed. Viruses CN738, CN739 and CN740 were generated with the same approach except that the CN251 (PB-TRE - E1A) plasmid was replaced with CN254 (PB-TRE - E1B), CN257 (PB-TRE - E1A, PSA-TRE - E1B), and CN273 (PSA-TRE - E1A, PB-TRE - E1B), respectively. In each of these viruses, the prostate-specific TRE (PB-TRE or PSA-TRE) was inserted between the  
15       appropriate coding segment (e.g., E1A or E1B) and its corresponding endogenous promoter. These viruses were therefore constructed by engineering one or two identical copies of the rat PB-TRE upstream of one or two essential adenovirus 5 early genes, E1A and E1B.

          In short, CN737 is an adenovirus type 5 which contains one PB-TRE upstream of  
20       the E1A gene; CN738, PB-TRE upstream of E1B; and CN740, two identical copies of PB-TRE upstream of the E1A and E1B.

### Example 2

#### 25           Testing a putative PB-TRE

          Plasmids similar to a reporter plasmid, such as CN280 described above, may be used to test the prostate-specificity of putative prostate-specific TREs (PS-TREs) or PS-TRE variants such as PB-TRE variants. The PS-TRE to be tested may have mutations  
30       such as deletions or insertions between binding sites known to be important in PS-TRE activity (e.g., are sites, etc.) or base substitutions in these sites themselves. For example,

some variants in the AREs (androgen receptor binding sites) of PB-TREs are known and described above and in the literature. Rennie et al. (1993) *Mol. Endocrinol.* 7:23-36.

Additional variant PB-TREs may comprise, for example, mutation(s) between a PB-TRE promoter and a PB-TRE enhancer (including deletions, substitutions and additions); a

- 5 combination of a non-prostate specific promoter and a PB-TRE enhancer; a combination of a non-prostate-specific promoter and a PSA-TRE enhancer; the rearrangement of segments of the enhancer and promoter of a prostate-specific TRE; or any other variant of a PB-TRE.

Briefly, the putative prostate-specific TRE or TRE variant such as a putative PB-TRE is inserted upstream of a reporter gene (exemplified by, but not limited to, firefly luciferase, *luc*). Reporter genes have been disclosed above, and methods for such construction are known in the art or are disclosed herein.

A comparison of reporter gene activity in prostate cells (or cells expressing prostate gene products) and non-prostate cells, using as a control an adenovirus with a non-cell-specific-promoter (e.g. CMV) controlling reporter gene expression, can indicate the efficacy of a putative PS-TRE in mediating prostate cell-specific gene expression.

### Example 3

#### Testing the cytotoxic ability of adenovirus vectors on prostate carcinoma cells and

#### tumor xenografts

##### **3. A. Experimental strategy**

An especially useful objective in the development of prostate-specific adenoviral vectors is to treat patients with prostate carcinoma. The strategy is to develop a prostate tissue-specific targeting pharmaceutical which could selectively kill a certain type of tumor cells (such as prostatic neoplasia) while leaving their non-cancerous neighbors unharmed. A 'smart bomb' virus, in which key gene expression is restricted by a tissue-specific regulatory element incorporated into its chromosome, meets this requirement. As stated previously, PB gene expression is exclusively in the prostate and is transcriptionally regulated by androgens. It has also been demonstrated that the minimal 454 nucleotides of PB-TRE is sufficient to target and restrict gene expression to prostate cells. This

arrangement limits the expression of important early viral genes to prostatic cells. The E1 coding segment, for example, can be placed under the control of PB-TRE; the TRE will remain silent in non-prostatic cell types and, consequently, prevent early gene expression, viral replication, and cell lysis. Several experiments have contributed to the understanding of PB-TRE-containing adenoviruses CN737, CN738 and CN740, described above.

An initial indicator of the feasibility is to test the vectors using a technique known in the art, such as testing the vectors for cytotoxicity against prostate and non-prostate cells *in vitro* (cytopathic effects), plaque assays, and tests against prostate carcinoma cells such as prostate xenografts grown subcutaneously in Balb/c nu/nu mice.

### **3. B. *In vitro* cytopathic effects of adenovirus with adenoviral early gene(s) under control of prostate-specific TRE(s)**

The first task at hand was to characterize the differential viral replication and cytopathic effects (CPE).

The adenovirus used included CN702 (full-length Ad5 with unaltered E1 region); CN737 (full-length Ad5 with E1A under control of PB-TRE; see Example 1) and CN740 (full-length Ad5 with both E1A and E1B under control of copies of PB-TRE; see Example 1).

CPE assays were performed as follows: Cells were infected with virus at increasing multiplicities of infection (MOI) and monitored for cytopathic effect. Ovarian carcinoma cells (OVCAR-3) and the human prostate cancer cells (LNCaP) each underwent complete monolayer cytolysis with wild-type adenovirus CN702 at MOI as low as 0.01 within 7 to 10 days. This indicated that wild-type adenovirus infects both prostatic and non-prostatic cells. In contrast, CN737 (PB-TRE - E1A) or CN740 (PB-TRE - E1A, PB-TRE - E1B)-infected LNCaP cells showed significant cytopathic effects at the same time points with MOI of 0.1 or 0.01. CN737 or CN740-infected OVCAR-3 cell monolayers did not show visible cytopathic effects with the same MOI.

Thus, adenoviruses in which one or two early Ad5 genes were under control of probasin TREs were able to mediate replication and cytopathic effects specifically in prostate cells, but not in non-prostate cells.

### 3. C. Plaque assay of adenovirus with adenovirus gene(s) under control of prostate-specific TRE(s)

The prostate-cell specificity of adenovirus viruses with adenovirus under control of prostate-specific TRE(s) was quantified. One method used to test this was a plaque assay.

- 5 A plaque assay is an infectious assay that quantifies how efficiently a particular virus produces a productive infection in a cell line. Plaques were evaluated in the following cell types: prostate cells (LNCaP) and non-prostate cells (MCF-7, BHL-100, OVCAR-3, and 293).

The plaque assay was performed as following: Confluent cell monolayers seeded in  
10 6-well dishes eighteen hours before infection were infected with 10-fold serial dilutions of CN737, CN738, CN740, and CN702. After infecting monolayers for four hours in serum-free media (MEM), the media was removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques were scored two weeks after infection.

15

*Table 1*  
*PB-TRE engineered adenovirus plaque assay*

Cell Line	Virus	Ave Titre	Titre/293	CN7--/CN702
293	CN702 (wt)	1.30E+06	1.0	
	CN737	3.40E+05	1.0	
	CN738	5.60E+05	1.0	
	CN740	4.80E+06	1.0	
LNCaP	CN702	1.40E+06	1.08	1.0
	CN737	1.90E+05	0.56	0.52
	CN738	4.00E+05	0.71	0.66
	CN740	1.50E+06	0.31	0.29
PC-3	CN702	1.80E+05	0.14	1.0
	CN737	1.10E+04	0.032	0.23
	CN738	5.00E+04	0.089	0.64
	CN740	4.50E+04	0.011	0.079
MCF-7	CN702	3.40E+05	0.26	1.0
	CN737	3.90E+02	0.0012	0.0046
	CN738	2.20E+04	0.065	0.25
	CN740	3.90E+03	0.00083	0.0032
HBL-100	CN702	2.00E+05	0.15	1.0
	CN737	1.40E+02	0.00041	0.0027
	CN738	8.60E+03	0.0150	0.1
	CN740	5.30E+01	0.000011	0.000073
OVCAR-3	CN702	4.10E+05	0.32	1.0
	CN737	1.40E+02	0.00041	0.0013
	CN738	1.40E+03	0.0025	0.0078
	CN740	1.20E+03	0.00025	0.00078

Table 1 shows the average titer of duplicate samples for the viruses tested. The  
5 titer for a particular virus in all cell lines was normalized to its titer on 293 cells. This  
allows comparisons between viruses in a particular cell type. Once the titers on a cell type  
were normalized to 293 cells, the normalized numbers of the recombinant viruses were  
compared to CN702. A ratio of less than one suggests that the virus tested plaques less

efficiently than CN702. Conversely, a ratio greater than one suggests that the virus plaques more efficiently than CN702.

Several interesting observations can be made from the plaque assay. First and most importantly, PB-TRE-engineered adenoviruses demonstrated preferential replication in prostate tumor cell line LNCaP compared to non-prostate cells. Since this carcinoma expresses androgen receptor and PSA, the PB regulatory region installed in the adenovirus vectors should be active in promoting the transcription of adenoviral early genes. The data suggests that this is the case, and the adenovirus comprising a PB-TRE induced cytopathic effects with an efficiency comparable to that of wild-type adenovirus in prostate tumor cells. Second, PB-TRE-driven adenoviruses show very little virus-mediated cytolysis of non-prostate tumor cells. The plaquing efficiency for three of the adenovirus vectors comprising a PB-TRE decreases dramatically in the non-prostate cell lines included in the experiment. Thus, PB-TRE engineered virus mediated cytolysis is significantly decreased relative to wild-type adenovirus in non-prostate cells.

Third, the vector CN740, in which two adenovirus replication genes (E1A and E1B) are controlled by separate PB-TREs, demonstrated unexpectedly better specificity than adenoviruses in which only one adenovirus gene was controlled by a PB-TRE. For example, in the AR-deficient cell line PC-3, CN737, in which a PB-TRE controls expression of E1A, showed a 4-fold increase in replication specificity compared to CN702 (wt). CN738, in which PB-TRE controls expression of E1B, showed little increase in specificity. However, CN740, in which PB-TREs control transcription of both E1A and E1B demonstrated a 13-fold increase in replication specificity, which is more than additive. Similar synergy was demonstrated with the AR-deficient cell line HBL-100. In this line, CN737 showed a 370-fold increase in specificity, and CN738, 10-fold. However, CN740 demonstrated a 14,000-fold increase in replication specificity. Therefore, the addition of a second prostate-specific TRE controlling an adenovirus replication gene results in a more than additive effect and an unexpectedly better increase in the cell-specific replication.

While all three vectors comprising a PB-TRE, CN737, CN738 and CN740 showed prostate-specific replication compared to wt CN702, CN737 and CN740 showed greater specificity than CN738 (PB-TRE - E1B).

In addition, in the prostate cell line, the PB-TRE-containing adenoviruses replicated slightly less well than wt CN702. For example, CN737 produced a titer of 0.56 relative to that of CN702. However, this reflects a small drop (up to 2- or 3-fold) in replication in prostate cells simultaneously obtained with a very large increase (up to 5 1,200-fold) in specificity.

In short, the PB-TRE adenoviruses described above demonstrated an ability to replicate specifically in prostate cells expressing androgen receptor.

### 3. D. Testing the efficacy against xenografts of adenovirus comprising an 10 adenovirus gene under control of a prostate-specific TRE

Mice are given subcutaneous injections with  $1 \times 10^7$  prostate carcinoma cells, such as LNCaP, in PBS. Tumor cells can be tested for probasin activity by assaying for probasin in serum using standard assays (for example, ELISA).

For this experiment, test virus vectors are introduced into the mice either by direct  
15 intratumoral, intravenous or intraperitoneal injection of approximately  $10^8$  pfu of virus (if administered as a packaged virus) in 0.1 ml PBS + 10% glycerol or intravenously via the tail vein. If administered as a polynucleotide construct (i.e., not packaged in virus), 0.1  $\mu$ g to 100  $\mu$ g or more can be administered. Tumor sizes are measured and, in some experiments, blood samples are taken weekly. The effect of intratumoral injection of an  
20 adenovirus vector of the present invention on tumor size and serum androgen receptor levels is compared to sham treatment.

While it is likely that a therapeutic based on the viruses described here would be given intralesionally (i.e., direct injection), it would also be desirable to determine if intravenous (IV) administration of the virus can affect tumor growth. If so, then it is  
25 conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. For this experiment, groups of five mice bearing prostate cancer tumors are inoculated with  $10^8$  pfu of an adenoviral vector of the present invention by tail vein injection, or  $10^8$  pfu of a replication defective adenovirus (CMV-LacZ) to control for non-specific toxic effects of the virus, or with buffer used to carry the virus. The effect of IV  
30 injection of the adenoviral vector on tumor size is compared to the sham treatment. As

shown in Example 5. F., the modified adenovirus of the present invention was capable of specifically eradicating prostate cell carcinoma induced in subject mice.

#### Example 4

##### 5     Construction of an adenoviral vector containing the coding region for the adenovirus death protein (ADP) under control of a prostate-specific TRE

An adenovirus vector in which the adenovirus death protein (ADP) was placed under control of a PB-TRE can be constructed as described below. ADP is encoded within  
10     the E3 region and naturally under control of the major late promoter (MLP). The gene appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement.

15     In an AR-specific viral vector (such as those described above in Example 1), a deletion can be created in the E3 region to accommodate a PB-TRE. The ADP coding sequence from Ad2 can be reintroduced into the E3 region of adenovirus Ad5 as follows:

An ADP cassette is constructed using overlap PCR. The Y leader, an important sequence for correct expression of some late genes, is PCR-amplified using primers:

20     5' GCCTTAATTAAGCAAACCTCACCTCCG...Ad2 28287bp (37.124.1)  
(SEQ ID NO:15); and

5' GTGGAACAAAAGGTGATTAAAAATCCCAG...Ad2 28622bp (37.146.1)  
(SEQ ID NO:16).

The ADP coding region is PCR amplified using primers

25     5' CACCTTTTGTCCACCGCTCTGCTTATTAC...Ad2 29195bp (37.124.3)  
(SEQ ID NO:17) and

5' GGCTTAATTAAGGTGAAAGGTGGGAGC...Ad2 29872bp (37.124.4) (SEQ ID NO:18).

The two fragments were annealed and the overlap product was PCR amplified  
30     using primers 37.124.1 and 37.124.4. The ends of the product were polished with Klenow fragment and ligated to BamHI cut pGEM-72 (+) (CN241; Promega, Madison, WI). The



ADP cassette was excised by digesting CN241 with *PacI* restriction endonuclease and ligated with two vectors, CN247 and CN248, generating plasmids CN252 and CN270, respectively. CN247 contains a unique *PacI* site in the E3 region and was constructed as follows. A plasmid containing the full length Ad5 genome, TG3602 (Transgene, France),  
5 was digested with *Bam*HI and religated to yield CN221. The backbone of this plasmid (outside of the adenovirus sequence) contained a *PacI* site that needed to be removed to enable further manipulations. This was effected by digesting CN221 with *PacI* and polishing the ends with T4 DNA polymerase, resulting in CN246. CN246 was digested with *AscI* and *AvrII* (to remove intact E3 region). This fragment was replaced by a  
10 similarly cut fragment derived from BHG11. The resulting plasmid, CN247, lacks the E3 region and a *PacI* site suitable for insertion of the ADP cassette fragment (described above). Ligation of CN247 with the ADP cassette generated CN252.

CN248 (a construct that would allow introduction of an ADP cassette into a Ad that also contains a deletion/substitution in the E4 region) was made as follows. The E4 region  
15 was deleted by digesting CN108, a construct that contains right hand end Ad5 sequence from the unique *EcoRI* site in the E3 region, with *AvrII* and *AflIII*. The only E4 ORF necessary for viral replication, ORF 6, was reintroduced by PCR amplifying the ORF with primers,

33.81.1 (Ad5 33096):

20 GCAGCTCACTTAAGTTCATGTCG (SEQ ID NO:19)

33.81.2 (Ad5 34084):

TCAGCCTAGGAAATATGACTACGTCCG (SEQ ID NO:20)

The resulting plasmid is CN203. CN203 was digested with *EcoRI* and ligated to CN209, a shuttle plasmid, to generate CN208. In the final cloning step, CN208 was  
25 digested with *AscI* and *AvrII* and ligated to similarly cut E4 deletion/substitution with the ADP cassette.

Thus, both CN252 and CN270 are adenoviral vectors containing the ADP gene and lacking the E3 gene. In addition, CN270 lacks some sequence in the E4 region as previously described. Full-length adenoviral vectors are obtained via *in vitro* ligation of  
30 (1) appropriately prepared viral DNA digested with *Bam*HI and (2) CN252 or CN257 also

digested with BamHI. The ligation product is used to transfect 293 cells. Plaque assays are performed as described in Example 1.

CN252 and CN270 can also be modified by insertion of a PB-TRE to place the ADP gene under transcriptional control of PB-TRE.

5

### Example 5

#### Additional analysis of adenoviruses in which adenovirus genes are under control of prostate-specific TREs

##### 10      **5. A. Adenoviral vectors**

In each of adenoviruses CN739 and CN753, two viral genes were under control of two separate prostate-specific TREs. These viruses were shown, as described below, to possess a stable genome; exhibit higher levels of specificity than adenoviruses in which only one gene was under control of a prostate-specific TRE; and mediate cell-specific  
15      adenoviral replication in cytopathic assays, plaque assays, and in *in vivo* tests on prostatic xenografts in athymic mice.

Adenovirus CN739, as described in Example 1.F., comprises a whole adenovirus in which a PB-TRE controls expression of E1A and PSA-TRE controls expression of E1B.

In adenovirus CN753, described in detail below, a PB-TRE controls expression of  
20      E1B, and a PSA-TRE controls E1A. In other adenovirus vectors, E1A was placed under control of a PB-TRE or PSA-TRE by inserting a PB-TRE or PSA-TRE between the native (wt) E1A promoter and the E1A coding segment, thus creating the sequence: native E1A promoter / PB-TRE (or PSA-TRE) / E1A coding segment. In CN753, the native E1A promoter was deleted, yielding the E1A coding segment under sole control of a prostate-  
25      specific enhancer.

CN753 was generated in several steps: The native E1A promoter was deleted (as a 64 bp fragment) from CN124 (wild-type left-hand end of Ad5) to generate CN306. The PSA-TRE fragment (from plasmid CN143, described above) was then inserted upstream of the promoter-less E1A coding segment of CN306 to generate CN321. A PB-TRE  
30      fragment (described above) was then inserted into the EagI site between the E1B promoter and E1B coding segment to generate CN326. CN326 thus has E1A under sole control of

PSA-TRE and E1B under control of PB-TRE. CN326 was then homologously recombined with BHG11, which contains the right hand side of Ad5 to generate CN753. The recombination was performed under a protocol similar to that outlined in 1. F.

5           **5. B.   Plaque assay demonstrating prostate cell specificity of adenovirus in which multiple adenoviral genes are under control of prostate-specific TREs**

Plaque assays were performed to determine the cell-specificity of adenoviruses in which multiple adenoviral genes are under control of prostate-specific TREs. A plaque assay is an infectious assay that quantifies how efficiently a particular virus produces an  
10 infection in a cell line. Plaquing efficiency was evaluated in the following cell types: prostate tumor cell lines (LNCaP, PC-3), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3), and human embryonic kidney cells (293).

The adenoviruses tested were:

- 15           CN702, wt, in which E1A and E1B are under control of their native promoters.  
          CN706, in which a PSA-TRE controls E1A expression.  
          CN739, in which a PB-TRE controls E1A expression and a PSA-TRE controls E1B.  
          CN753, in which a PSA-TRE controls E1A, and a PB-TRE controls E1B.

20           The plaque assay was performed as follows: Confluent cell monolayers were seeded in 6 well dishes eighteen hours before infection. The monolayers were infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the media was removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques were scored two weeks after  
25 infection. CN702 has no modifications in its E1 region and is used as a wild-type control.

*Table 2*  
*PSA-TRE and PB-TRE engineered adenovirus plaque assay data.*

5 *Percent of wild-type adenovirus (PFU/ml)*

	Cell Lines			
	293	LNCaP	HBL-100	OVCAR-3
<b>Viruses</b>				
CN702	100	100	100	100
CN706	100	33	2.5	3.4
CN739	100	35	0.12	0.0023
CN753	100	41	0.23	0.11

Table 2 shows the average titer of duplicate samples for the viruses tested. The  
 10 titer for a particular virus in all cell lines was normalized to its titer on 293 cells. This  
 allows comparisons to be made between viruses in a particular cell type. Once the titers on  
 a cell type were normalized to 293 cells, the normalized numbers of the recombinant  
 viruses were compared to CN702. A ratio of less than 100 suggests that the virus tested  
 plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the  
 15 virus plaques more efficiently than CN702.

Several interesting observations can be made from this plaque assay. First,  
 adenoviruses in which two adenoviral replication genes are controlled by a PSA-TRE and  
 a PB-TRE demonstrated replication in prostate tumor cells, although this replication shows  
 a slightly lower efficiency than wild type adenovirus.

20 Second, adenoviruses in which two adenoviral replication genes are controlled by  
 PSA-TRE and PB-TRE demonstrated an unexpectedly high preferential replication in  
 prostate tumor cells. The virus CN706, in which a PSA-TRE controls E1A, showed a 29-  
 fold increase in replication cell-specificity. When two prostate-specific promoters, a PSA-  
 TRE and a PB-TRE, controlled transcription of E1A and E1B, the specificity increased  
 25 800-fold (for CN753) or over 40,000-fold in OVCAR-3 cells. Similarly, the adenoviruses  
 comprising two prostate-specific TREs demonstrated significantly better specificity of  
 replication in HBL-100 cells. This increase in specificity is more than an additive effect of  
 inserting a second prostate-specific TRE.

A significant result shown in Table 2 was that viral replication of CN739 proved much more specific than that of CN753. In both of these viruses, two adenovirus replication genes, E1A and E1B, are controlled by prostate-specific TREs, a PB-TRE or a PSA-TRE. E1A is arguably more important for viral replication than E1B, as E1A is  
5 expressed before any other viral genes, including E1B, and is required for E1B expression. Flint (1982); Flint (1986); Grand (1987). CN753, in which a PSA-TRE controls expression of E1A, demonstrated an 800-fold increase in cell-specific replication in OVCAR-3 cells. CN739, in which PB-TRE controls expression of E1A, showed a greater  
10 40,000-fold increase. This result suggests that, under at least some conditions, PB-TRE has a much greater ability to regulate cell-specific replication than a TRE derived from the prostate specific antigen gene.

Third, PSA-TRE and PB-TRE viruses give 10- to 100-fold less plaques in HBL-100 and OVCAR-3 cells than CN706, but their titers were similar to CN706 in LNCaP cells. PSA-TRE and PB-TRE engineered viruses were significantly attenuated relative to  
15 wild-type adenovirus and CN706 in non-prostate cells, but they showed similar activity to CN706 in LNCaPs. While the adenoviruses containing prostate-specific TREs showed an ability to replicate in prostate cells decreased up to three-fold, this occurred simultaneously with the achievement of a greatly increased specificity (up to 1,000-fold).

Thus, an adenovirus in which one adenoviral gene was under control a prostate-specific TRE showed great specificity to prostate cells, but adenoviruses in which two  
20 adenoviral genes were under control of two separate prostate-specific promoters showed even greater specificity, in plaque assays.

#### **5. C. Cytopathic effects assay demonstrating prostate cell specificity of 25 adenovirus in which multiple adenoviral genes are under control of prostate-specific TREs**

The cytopathic effects assay (CPE) was also used to determine the cell-specificity of replication of adenoviruses in which multiple adenoviral genes were placed under control of prostate-specific TREs.

30 The adenoviruses tested were:

CN702, in which E1A and E1B are under control of their native promoters; and

CN739, in which PB-TRE controls E1A expression and PSA-TRE controls E1B.

CPE assays were performed as follows: Cells were infected with virus at increasing multiplicities of infection (MOI) and monitored for cytopathic effect. Assays were terminated when complete cytolysis of the monolayers was observed at an MOI of 5 0.01 with wild-type adenovirus. One primary, non-immortalized human microvascular endothelial cell line was chosen to test its sensitivity to CN739 and wild-type adenovirus (CN702) infection, *in vitro*. CN702 caused complete monolayer cytolysis of hMVECs (primary human microvascular endothelial cells) at MOIs as low as 0.01 within 10 days. In contrast, CN739-infected hMVEC monolayers did not show significant cytopathic 10 effects at the same time points with MOIs of 10, 1.0, 0.1 and 0.01. Cytolysis of hMVECs equivalent to that seen with wild-type adenovirus was only evident at MOIs between 100 and 1000 times as high (MOI > 10).

Thus, CN739-mediated cytolysis is significantly attenuated relative to wild-type adenovirus in primary normal human cells.

15

#### 5. D. Differential viral replication

In order to determine if levels of virus replication correlate with the cytopathic effects of CN739 in prostate tumor cells or human normal cells, we carried out virus 20 replication titration on PSA-producing prostate tumor cells (LNCaP) and non-prostate cells, primary human microvascular endothelial cells (hMVECs). Cells were grown to 70-90% confluence and infected with either wild-type adenovirus (CN702) or CN739 for 90 min at a MOI of 10. Fifty-five hours after infection, the virus was released from the cells by three freeze/thaw cycles, and the resulting supernatant was titered on 293 cells. The 25 amount of CN739 produced was normalized against the amount of wild-type virus produced in the same cell line during the same time period. The data shown in Figure 6 indicate that CN739 titers were 30% of CN702 titers in LNCaPs, but were reduced to less than 1/100th those of the wild-type virus in normal (hMVEC) cells. This suggests that CN739 replicates poorly in primary normal human cells, and is only slightly attenuated in 30 prostate cancer cells.

In short, an adenovirus in which multiple adenoviral genes were under control of prostate-specific TREs demonstrated replication in prostate cells, and only very poor replication in non-prostate endothelial cells.

5           **5. E. One-step growth curve**

A one-step growth curve assay was used to determine the efficiency and kinetics of replication of various adenoviruses in prostate and non-prostate endothelial cells. Viruses used were CN702 (wt), CN706 (PSA-TRE controlling E1A expression), and CN739 (PB-TRE controlling E1A and PSA-TRE controlling E1B). Cells used were LNCaPs (prostate  
10 tumor cells) and hMVECs (primary human microvascular endothelial cells). Replicate monolayers of LNCaPs and hMVECs were infected at a MOI of 10 to obtain a synchronous infection of all the cells. Duplicate cultures were harvested at various times post-infection. The number of infectious virus was determined by plaque assay on 293 cells (Figure 7). CN739 and CN706 grew at a similar efficiency in LNCaPs. However,  
15 under identical conditions, CN739 grew poorly in the hMVECs, producing about 10,000-fold and 80,000-fold less infectious virus than CN706 and wild-type adenovirus, respectively.

Thus, the one-step growth curve demonstrated that viruses in which multiple adenoviral genes were under control of prostate-specific TREs grew well in prostate tumor  
20 cells, but grew very poorly in non-prostate endothelial cells, indicating significantly enhanced specificity.

**5. F. Therapeutic effect studies**

25           The efficacy of treating prostate tumors *in vivo* was tested with an adenovirus in which multiple genes were under control of two separate prostate-specific TREs, a PB-TRE and a PSA-TRE (CN739). To examine the therapeutic efficacy of CN739 *in vivo*, LNCaP (prostate tumor) xenografts were grown in athymic mice. The tumor cells were injected subcutaneously into each flank of each mouse, and after establishment of palpable  
30 tumors (mean tumor volume 300 mm<sup>3</sup>), the tumors were directly injected with CsCl-purified CN739 at 2.5 x 10<sup>8</sup> particles per mm<sup>3</sup>, or PBS containing 10% glycerol (vehicle)

as a control. Tumor growth was then followed for 6 weeks, at which time the mean tumor volume in each group was determined and serum samples were collected for PSA analysis on day 0 and weekly thereafter.

The data depicted in Figure 9 show that treatment of LNCaP tumors with CN739  
5 resulted in an 80% reduction in average tumor volume whereas the average tumor volume in vehicle-treated group I, at day 28, had increased to 400% of the initial volume. Four of seven (57%) animals in CN739-treated group II were free of palpable tumors at day 42. This study demonstrates that a fixed, single dose of an adenovirus in which multiple genes are under control of prostate-specific TREs (CN739) per tumor is efficacious against  
10 LNCaP xenografts *in vivo*.

#### 5. G. Effects on serum PSA levels

The serum PSA level is a widely-used marker for the diagnosis and management of prostate carcinoma. LNCaP cells express and secrete high levels of PSA into the culture  
15 media and into circulation. An experiment was designed to examine the effects of treatment with CN739 (an adenovirus in which multiple genes were under control of prostate-specific TREs PB-TRE and PSA-TRE) on the serum PSA concentration in mice with LNCaP tumor xenografts.

Following treatment, the average serum PSA level in control group I (vehicle only)  
20 increased to approximately 800% of the initial value by day 28, whereas the average PSA level in group II (CN706 treatment) and group III (CN739 treatment) remained essentially constant through day 21 and declined to 10% of the initial value by day 35 (Figure 8). There was a statistically significant difference ( $p < 0.001$ ; T-test) between group I and group II on day 14 and thereafter.

25 These results demonstrate that CN739 treatment is efficacious in the LNCaP xenograft model when the outcome is measured either by reduction in tumor growth or serum PSA concentration. Taken together with the *in vitro* data, it suggests that adenoviruses with a single adenoviral gene under control of a prostate-specific TRE such as PB-TRE are able to mediate prostate cell-specific viral replication. Placing a second  
30 adenoviral gene under control of a separate prostate-specific TRE further increases the cell-specificity.



### Example 6

#### Characterization of an E3 deleted adenovirus, CN751, that contains the adenovirus death protein gene

5

An adenovirus comprising an adenovirus death protein, CN751, was constructed to test whether such a construct may be more effective for cytotoxicity. The adenovirus death protein (ADP), an 11.6-kDa Asn-glycosylated integral membrane peptide expressed at high levels late in infection, migrates to the nuclear membrane of infected cells and affects efficient lysis of the host. The Adenovirus 5 (Ad5) E3 region expresses the *adp* gene.

#### *Construction of CN751*

15 CN751 was constructed in two parts. First, an E3 deleted platform plasmid that contains Ad5 sequence 3' from the BamHI site at 21562bp was generated. The Ad2 *adp* was engineered into the remainder of the E3 region of this plasmid to yield CN252 (this cloning has been previously described). To construct the second part, the 5' Ad5 sequence necessary for CN751 was obtained by digesting purified CN702 DNA with EcoRI and  
20 isolating the left hand fragment by gel extraction. After digesting CN252 with EcoRI, the left hand fragment of CN702 and CN252 were ligated. 293 cells were transfected with this ligation mixture by lipofection transfection and incubated at 37°C. Ten days later, the cells were harvested, freeze-thawed three times, and the supernatant was plaqued on 293 monolayers. Individual plaques were picked and used to infect monolayers of 293 cells to  
25 grow enough virus to test. After several days, plate lysates were screened using a polymerase chain reaction (PCR) based assay to detect candidate viruses. One of the plaques that scored positive was designated CN751.

#### *Structural Characterization of CN751*

30 The structure of CN751 was confirmed by two methods. First, primers 37.124.1 (5' gccttaattaaaagcaaacctcacctccg Ad2 28287bp) and 37.124.4 (5'

ggcttaattaactgtgaaaggtgggctgc Ad2 29872bp) were used to screen candidate viruses by PCR to detect the presence of the *adp* cassette. CN751 produced an extension fragment consistent with the expected product (1065bp). Second, CN751 was analyzed by Southern blot. Viral DNA was purified, digested with *PacI*, *SacI*, and *AccI/XhoI*, and probed with a  
5 sequence homologous to the ADP coding region. The structure of CN751 matched the expected pattern.

### *In Vitro Characterization of CN751*

Two experiments were conducted to examine the cytotoxicity and virus yield of  
10 CN751. In the first study, CN751's cytotoxicity was evaluated in LNCaP cells by measuring the accumulation of a cytosolic enzyme, lactate dehydrogenase (LDH), in the supernatant over several days. The level of extracellular LDH correlates with the extent of cell lysis. Healthy cells release very little, if any, enzyme, whereas dead cells release large quantities. LDH was chosen as a marker because it is a stable protein that can be readily  
15 detected by a simple protocol. CN751's ability to cause cell death was compared to that of CN702, a vector lacking the ADP gene, and Rec700, a vector containing the ADP gene.

Monolayers of LNCaP cells were infected at an MOI of one with either CN702, Rec700 (*adp*+ control), or CN751 and then seeded in 96 well dishes. Samples were harvested once a day from one day after infection to five days after infection and scored  
20 using Promega's Cytotox 96 kit. This assay uses a coupled enzymatic reaction which converts a tetrazolium salt to a red formazan product that can be determined in a plate reader at 490nm.

Since the absorbance of a sample corresponds to the level of LDH released from infected cells, a plot of how a sample's absorbance changes with time describes how  
25 efficiently the viruses studied induce cell lysis (Figure 10). Each data point represents the average of sixteen separate samples. The results suggest that CN751 kills cells more efficiently than the *adp*- control, CN702, and similarly to the *adp*+ control, Rec700. The concentration of LDH in the supernatant increases rapidly from two days and reaches a maximum at four days in wells infected with CN751. In contrast, LDH concentration in  
30 the supernatant of CN702 infected cells begins to rise slowly at two days and continues until the conclusion of the experiment. Significantly, the amount of LDH released from

CN751 infected cells at three days is two times that released from CN702 infected cells. In sum, the virus yield data demonstrate that adenoviral vectors with the ADP gene release more virus.

Not only is it important for Ad vectors to kill cells efficiently, they must also be able to shed progeny that can infect other cancer cells. Viral vectors that can shed large amounts of virus might be better therapeutics than those that shed only small amounts. A virus yield assay was undertaken to evaluate whether CN751 can induce the efficient release of its progeny from the infected cell. A549 cells were infected at an MOI of five. Supernatant was harvested at various times after infection and titered on 293 cells to determine the virus yield (Figure 11). The data suggest that cells infected with CN751 shed virus more efficiently than those infected with CN702. At forty-eight hours post infection, CN751 infected cells released ten times more virus than CN702 infected. At seventy-two hours post infection, CN751 infected cells released forty times more virus. The data demonstrate that adenoviral vectors with the ADP gene kill cells more efficiently than adenoviral vectors that lack the ADP gene.

#### *In vivo characterization of CN751*

LNCaP nude mouse xenografts were challenged with a single intratumoral dose ( $1 \times 10^4$  particles/mm<sup>3</sup> tumor) of either CN751, a vector containing the ADP gene, or CN702, a vector lacking the gene. A third group of tumors was treated with buffer alone. The tumors were monitored weekly for six weeks and their relative volume was graphed against time. The results are shown in Figure 12. Error bars represent the standard error for each sample group. The initial average tumor volume for CN751 treated animals (n = 14) was 320 mm<sup>3</sup> for CN702 treated (n = 14), and 343 mm<sup>3</sup> for buffer treated (n = 8). The data suggest that CN751 kills tumor cells more effectively than CN702. On average, tumors challenged with CN751 remained the same size throughout the course of the experiments while nine out of fourteen tumors (64%) regressed. Those treated with CN702 doubled in size. Buffer treated tumors grew to nearly five times their initial volume. The Students T-test indicates that the difference in tumor size between CN751 and CN702 treated tumors was statistically significant from day 7 (p = 0.016) through the end of the experiment (p = 0.003).

Example 7A PB-TRE demonstrates higher cell-specificity of replication  
than a human glandular kallikrein-1 TRE**6. A. Adenoviral vectors used in this example include:**

CN702 (wt);

CN706, in which a PSA-TRE controls E1A expression;

10 CN753, in which a PSA-TRE controls E1A expression and a PB-TRE controls  
E1B; and

CN755, in which a PSA-TRE controls E1A expression and a hKLK2-TRE (from  
the human glandular kallikrein-1 gene) controls E1B expression.

15 Plaque assays were performed to determine the cell-specificity of these  
adenoviruses. Plaquing efficiencies were evaluated on the following cell types: human  
embryonic kidney cell line (293), prostate tumor cell line (LNCaP), breast normal cell line  
(HBL-100), and ovarian tumor cell line (OVCAR-3). The plaque assays were performed  
as described above.

20 *Table 3*  
*PSA-TRE-, PB-TRE- and hKLK2-engineered adenovirus plaque assay data.*

*Percent of wild-type adenovirus (PFU/ml)*

	Cell Lines			
	293	LNCaP	HBL-100	OVCAR-3
Viruses				
CN702	100	100	100	100
CN706	100	25	2.7	7.7
CN753	100	33	0.067	0.11
CN755	100	29	0.52	0.6

25 Table 3 shows the average titer of samples for the viruses tested. The titer for a  
particular virus in all cell lines was normalized to its titer on 293 cells. This allows  
comparisons to be made between viruses in a particular cell type. Once the titers on a cell  
type were normalized to 293 cells, the normalized numbers of the recombinant vectors

were compared to CN702. A ratio of less than 100 suggests that the virus tested plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the virus plaques more efficiently than CN702.

Several interesting observations can be made from this plaque assay. First, this  
5 assay reiterates the unexpected finding described above that an adenovirus in which more than one adenovirus gene is under control of a prostate-specific TRE displays much a more than additive cell-specificity than an adenovirus in which only one adenovirus gene is under control of a prostate-specific TRE.

Second, CN753 demonstrates a significantly higher cell-specificity than CN755. In  
10 both CN753 and CN755, a PSA-TRE controls expression of E1A. However, in CN753, a PB-TRE controls expression of E1B, while in CN755, an hKLK2-TRE controls E1B. CN753 demonstrates a 5-fold higher cell-specificity in ovarian cells, and an 8-fold higher specificity in breast cells. Thus, it appears that, while both a PB-TRE and an hKLK2-TRE are thought to be specific for prostate cells, a PB-TRE, under at least some conditions, has  
15 a significantly higher specificity to prostate cells than an hKLK2-TRE.

### Example 8

#### Preparation of Covalently Pegylated Adenovirus

20 A series of experiments were carried out to alter the surface capsids of adenovirus by complexing adenovirus with PEG. The objective of PEG complexation masking of the adenovirus surface is the following: 1) adenovirus neutralizing antibodies or opsinins which are in circulation, and 2) increase systemic circulation time of adenovirus particles by reduction of non-specific clearance mechanism in the body (i.e., macrophages, etc.).

25 Surface capsids of wild type adenovirus CN706 were modified through covalent attachment of PEG to hexon and fiber proteins using N-hydroxysuccinimidyl succinamide (NHS). The PEGs (Shearwater Polymers, Inc.) had nominal molecular weight of 5000 Da. The activated PEG (approximately 2 mM) was reacted with  $5 \times 10^9$  particles/ml adenovirus in a tris-HCl buffer (the approximate molar ratio of virus particle to PEG was  $1:4 \times 10^6$ ).

30 Various combinations of pH, temperature, and reaction times were used. After the reaction, unreacted activated PEG, unreacted adenovirus, and pegylated adenovirus were

separated by anionic ion exchange chromatography on Q Sepharose XL (Pharmacia), running a 0 to 1.5 M NaCl gradient in 50 mM tris, pH 8.0. The gradient was run over 10 column volumes.

#### *Characterization of PEG-CN706*

5       Pegylation of CN706 was verified by SDS-Page. Figure 14 depicts the pegylation of CN706 and the mobility shift of pegylated proteins. Lanes 1 and 2 are non-pegylated CN706 (control), lanes 3 through 6 are pegylated CN706 under several pH and temperature conditions. Lanes 3 through 6 show the appearance of a second band above the hexon proteins of CN706, most likely pegylated hexon, and the loss of the fiber protein band.  
10    Since no additional bands associated with the virus except that corresponding to the PEG-hexon protein, the pegylated fiber protein is assumed to be under one of the unpegylated proteins on the SDS gel.

Figure 15 is an ion exchange chromatogram showing the change in surface properties of CN706. Pegylation of CN706 results in its earlier elution from the Q  
15    Sephadex resin used to capture the virus. This result is most likely due to PEG rendering the virus more charge neutral in appearance and hence decreasing its binding potential to the ion exchange matrix. A broadening of the virus' chromatogram is expected since the pegylation of CN706 occurs to different percentages.

The infectivity of pegylated CN706 was evaluated in an *in vitro* plaque assay on  
20    293 cells. Table 4 depicts a 5 to 10-fold reduction in plaquing efficiency of PEG-CN706 as compared to CN706. This is most likely due to pegylation masking the virus cells, decreasing the recognition and endocytosis of the viral particles.

Table 4: Comparison of Plaquing Efficiency of CN706 and PEG-CN706.

25	Sample Description	Number of Plaques (Arbitrary Units)
	CN706	15 $\pm$ 5
	PEG-CN706	4 $\pm$ 1

30       Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to

those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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  - (E) COUNTRY: USA
  - (F) ZIP: 94304-1018
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: Windows
  - (D) SOFTWARE: FastSEQ for Windows Version 2.0b
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 02-MAR-1998
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Catherine, Polizzi M
  - (B) REGISTRATION NUMBER: 40,130
  - (C) REFERENCE/DOCKET NUMBER: 34802-20007.00
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 650-813-5600
  - (B) TELEFAX: 650-494-0792
  - (C) TELEX: 706141

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 454 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTCCAC	AAGTGCATTT	AGCCTCTCCA	GTATTGCTGA	TGAATCCACA	GTTCAGGTTT	60
AATGGCGTTC	AAAACCTTGAT	CAAAAATGAC	CAGACTTTAT	ATTCTTACAC	CAACATCTAT	120
CTGATTGGAG	GAATGGATAA	TAGTCATCAT	GTTTAAACAT	CTACCATTCC	AGTTAAGAAA	180
ATATGATAGC	ATCTTGTTCT	TAGTCTTTTT	CTTAATAGGG	ACATAAAGCC	CACAAATAAA	240
AATATGCCTG	AAGAATGGGA	CAGGCATTGG	GCATTGTCCA	TGCCTAGTAA	AGTACTCCAA	300
GAACCTATTT	GTATACTAGA	TGACACAATG	TCAATGTCTG	TGTACAAC TG	CCAACTGGGA	360
TGCAAGACAC	TGCCCATGCC	AATCATCCTG	AAAAGCAGCT	ATAAAAAGCA	GGAAGCTACT	420
CTGCACCTTG	TCAGTGAGGT	CCAGATACCT	ACAG			454

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5836 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAGCTTCTAG	TTTTCTTTTC	CCGGTGACAT	CGTGGAAAGC	ACTAGCATCT	CTAAGCAATG	60
ATCTGTGACA	ATATTCACAG	TGTAATGCCA	TCCAGGGAAC	TCAACTGAGC	CTTGATGTCC	120
AGAGATTTTT	GTGTTTTTTT	CTGAGACTGA	GTCTCGCTCT	GTGCCAGGCT	GGAGTGCACT	180
GGTGCAACCT	TGGCTCACTG	CAAGCTCCGC	CTCCTGGGTT	CACGCCATTC	TCCTGCCTCA	240
GCCTCCTGAG	TAGCTGGGAC	TACAGGCACC	CGCCACCACG	CCTGGCTAAT	TTTTTTGTAT	300
TTTTAGTAGA	GATGGGGTTT	CACGTGTGTTA	GCCAGGATGG	TCTCAGTCTC	CTGACCTCGT	360
GATCTGCCCA	CCTTGGCCTC	CCAAAGTGCT	GGGATGACAG	GCGTGAGCCA	CCGCGCCTGG	420
CCGATATCCA	GAGATTTTTT	GGGGGGCTCC	ATCACACAGA	CATGTTGACT	GTCTTCATGG	480
TTGACTTTTA	GTATCCAGCC	CCTCTAGAAA	TCTAGCTGAT	ATAGTGTGGC	TCAAAACCTT	540
CAGCACAAT	CACACCGTTA	GACTATCTGG	TGTGGCCCAA	ACCTTCAGGT	GAACAAAGGG	600
ACTCTAATCT	GGCAGGATAT	TCCAAAGCAT	TAGAGATGAC	CTCTTGCAAA	GAAAAAGAAA	660
TGGAAGAGAA	AAAGAAAGAA	AGGAAAAAAA	AAAAAAGAAA	GAGATGACCT	CTCAGGCTCT	720
GAGGGGAAAC	GCCTGAGGTC	TTTGAGCAAG	GTCAGTCCTC	TGTTGCACAG	TCTCCCTCAC	780
AGGGTCATTG	TGACGATCAA	ATGTGGTCAC	GTGTATGAGG	CACCAGCACA	TGCCTGGCTC	840
TGGGGAGTGC	CGTGTAAGTG	TATGCTTGCA	CTGCTGAATG	CTTGGGATGT	GTGAGGGATT	900
ATCTTCAGCA	CTTACAGATG	CTCATCTCAT	CCTCACAGCA	TCACTATGGG	ATGGGTATTA	960
CTGGCCTCAT	TTGATGGAGA	AAGTGGCTGT	GGCTCAGAAA	GGGGGGACCA	CTAGACCAGG	1020
GACACTCTGG	ATGCTGGGGA	CTCCAGAGAC	CATGACCACT	CACCAACTGC	AGAGAAATTA	1080
ATTGTGGCCT	GATGTCCCTG	TCCTGGAGAG	GGTGGAGGTG	GACCTTCACT	AACCTCCTAC	1140
CTTGACCTC	TCTTTTAGGG	CTCTTTCTGA	CCTCCACCAT	GGTACTAGGA	CCCCATTGTA	1200
TTCTGTACCC	TCTTGACTCT	ATGACCCCCA	CTGCCCACTG	CATCCAGCTG	GGTCCCCTCC	1260
TATCTCTATT	CCCAGCTGGC	CAGTGCAGTC	TCAGTGCCCA	CCTGTTTGTC	AGTAACTCTG	1320
AAGGGGCTGA	CATTTTACTG	ACTTGCAAAC	AAATAAGCTA	ACTTTCCAGA	GTTTTGTGAA	1380
TGCTGGCAGA	GTCCATGAGA	CTCCTGAGTC	AGAGGCCAAG	GCTTTTACTG	CTCACAGCTT	1440
AGCAGACAGC	ATGAGGTTCA	TGTTACATT	AGTACACCTT	GCCCCCCCCA	AATCTTGTAG	1500
GGTGACCAGA	GCAGTCTAGG	TGGATGCTGT	GCAGAAGGGG	TTTGTGCCAC	TGGTGAGAAA	1560
CCTGAGATTA	GGAATCCTCA	ATCTTATACT	GGGACAACCT	GCAAACCTGC	TCAGCCTTTG	1620
TCTCTGATGA	AGATATTATC	TTCATGATCT	TGGATTGAAA	ACAGACCTAC	TCTGGAGGAA	1680
CATATTGTAT	CGATTGTCTT	TGACAGTAAA	CAAACTGTGT	GTAAGAGACA	TTATCTTTAT	1740
TATCTAGGAC	AGTAAGCAAG	CCTGGATCTG	AGAGAGATAT	CATCTTGCAA	GGATGCTTGC	1800
TTTACAAACA	TCCTTGAAAC	AACAATCCAG	AAAAAAGGAG	GTGTTGCTGT	CTTTGCTCAG	1860
AAGACACACA	GATACGTGAC	AGAACCATGG	AGAATTGCCT	CCCAACGCTG	TTCAGCCAGA	1920

GCCTTCCACC	CTTGTCTGCA	GGACAGTCTC	AACGTTCCAC	CATTAAATAC	TTCTTCTATC	1980
ACATCCTGCT	TCTTTATGCC	TAACCAAGGT	TCTAGGTCCC	GATCGACTGT	GTCTGGCAGC	2040
ACTCCACTGC	CAAACCCAGA	ATAAGGCAGC	GCTCAGGATC	CCGAAGGGGC	ATGGCTGGGG	2100
ATCAGAACTT	CTGGGTTTGA	GTGAGGAGTG	GGTCCACCCT	CTTGAATTTT	AAAGGAGGAA	2160
GAGGCTGGAT	GTGAAGGTAC	TGGGGGAGGG	AAAGTGTGAG	TTCCGAATC	TTAGGTCAAT	2220
GAGGGAGGAG	ACTGGTAAGG	TCCCAGCTCC	CGAGGTACTG	ATGTGGGAAT	GGCCTAAGAA	2280
TCTCATATCC	TCAGGAAGAA	GGTGCTGGAA	TCCTGAGGGG	TAGAGTTCTG	GGTATATTTG	2340
TGGCTTAAGG	CTCTTTGGCC	CCTGAAGGCA	GAGGCTGGAA	CCATTAGGTC	CAGGGTTTGG	2400
GGTGATAGTA	ATGGGATCTC	TTGATTCCCT	AAGAGTCTGA	GGATCGAGGG	TTGCCCATTG	2460
TTCCATCTTG	CCACCTAATC	CTTACTCCAC	TTGAGGGTAT	CACCAGCCCT	TCTAGCTCCA	2520
TGAAGGTCCC	CTGGGCAAGC	ACAATCTGAG	CATGAAAGAT	GCCCCAGAGG	CCTTGGGTGT	2580
CATCCACTCA	TCATCCAGCA	TCACACTCTG	AGGGTGTGGC	CAGCACCATG	ACGTATGTGT	2640
GCTGTGACTA	TCCCTGCAGC	GTGCCTCTCC	AGCCACCTGC	CAACCGTAGA	GCTGCCCATC	2700
CTCCTCTGGT	GGGAGTGGCC	TGCATGGTGC	CAGGCTGAGG	CCTAGTGTCA	GACAGGGAGC	2760
CTGGAATCAT	AGGGATCCAG	GACTCAAAAG	TGCTAGAGAA	TGGCCATATG	TCACCATCCA	2820
TGAAATCTCA	AGGGCTTCTG	GGTGGAGGGC	ACAGGGACCT	GAACCTATGG	TTTCCCAAGT	2880
CTATTGCTCT	CCCAAGTGAG	TCTCCAGAT	ACGAGGCACT	GTGCCAGCAT	CAGCCTTATC	2940
TCCACCACAT	CTTGTAAGAG	GACTACCCAG	GGCCCTGATG	AACACCATGG	TGTGTACAGG	3000
AGTAGGGGGT	GGAGGCACGG	ACTCCTGTGA	GGTCACAGCC	AAGGGAGCAT	CATCATGGGT	3060
GGGGAGGAGG	CAATGGACAG	GCTTGAGAAC	GGGGATGTGG	TTGTATTTGG	TTTTCTTTGG	3120
TTAGATAAAG	TGCTGGGTAT	AGGATTGAGA	GTGGAGTATG	AAGACCAGTT	AGGATGGAGG	3180
ATCAGATTGG	AGTTGGGTGA	GATAAAGTGC	TGGGTATAGG	ATTGAGAGTG	GAGTATGAAG	3240
ACCAGTTAGG	ATGGAGGATC	AGATTGGAGT	TGGGTTAGAG	ATGGGGTAAA	ATTGTGCTCC	3300
GGATGAGTTT	GGGATTGACA	CTGTGGAGGT	GGTTTGGGAT	GGCATGGCTT	TGGGATGGAA	3360
ATAGATTTGT	TTTGATGTTG	GCTCAGACAT	CCTTGGGGAT	TGAAGTGGGG	ATGAAGCTGG	3420
GTTTGATTTT	GGAGGTAGAA	GACGTGGAAG	TAGCTGTGAG	ATTTGACAGT	GGCCATGAGT	3480
TTTGTTTGAT	GGGGAATCAA	ACAATGGGGG	AAGACATAAG	GGTTGGCTTG	TTAGGTTAAG	3540
TTGCGTTGGG	TTGATGGGGT	CGGGGCTGTG	TATAATGCAG	TTGGATTGGT	TTGTATTAAA	3600
TTGGGTTGGG	TCAGGTTTTG	GTGAGGATG	AGTTGAGGAT	ATGCTTGGGG	ACACCGGATC	3660
CATGAGGTTT	TCACTGGAGT	GGAGACAAAC	TTCTTTTCCA	GGATGAATCC	AGGGAAGCCT	3720
TAAATTCACG	GTAGGGGAGG	TCAGGCCACT	GGCTAAGTAT	ATCCTTCCAC	TCCAGCTCTA	3780
AGATGGTCTT	AAATTTGTGAT	TATCTATATC	CACCTTCTGTC	TCCCTCACTG	TGCTTGGAGT	3840
TTACCTGATC	ACTCAACTAG	AAACAGGGGA	AGATTTTATC	AAATTCCTTT	TTTTTTTTTT	3900
TTTTTTTTTG	GACAGAGTCT	CACCTCTGTT	CCCAGGCTGG	AGTGCAGTGG	CGCAGTCTCG	3960
GCTCACTGCA	ACCTCTGCCT	CCCAGGTTCA	AGTGATTTCT	CTGCCCTCAG	CTCCTGAGTT	4020
GCTGGGATTA	CAGGCATGCA	GCACCATGCC	CAGCTAATTT	TTGTATTTTT	AGTAGAGATG	4080
GGGTTTCACC	AATGTTTGCC	AGGCTGECCT	CGAACTCCTG	ACCTGGTGAT	CCACCTGCCT	4140
CAGCCTCCCA	AAGTGCTGGG	ATTACAGGCG	TCAGCCACCG	CGCCCAGCCA	CTTTTGTCAA	4200
ATTCTTGAGA	CACAGCTCGG	GCTGGATCAA	GTGAGCTACT	CTGGTTTTAT	TGAACAGCTG	4260
AAATAACCAA	CTTTTTGGAA	ATTGATGAAA	TCTTACGGAG	TTAACAGTGG	AGGTACCAGG	4320
GCTCTTAAGA	GTTCCCGATT	CTCTTCTGAG	ACTACAAATT	GTGATTTTGC	ATGCCACCTT	4380
AATCTTTTTT	TTTTTTTTTT	TAAATCGAGG	TTTCAGTCTC	ATTCTATTTT	CCAGGCTGGA	4440
GTTCAATAGC	GTGATCACAG	CTCACTGTAG	CCTTGAACCT	CTGGCCTTAA	GAGATTCTCC	4500
TGCTTCGGTC	TCCCAATAGC	TAAGACTACA	GTAGTCCACC	ACCATATCCA	GATAATTTTT	4560
AAATTTTTTG	GGGGGCCGGG	CACAGTGGCT	CACGCTGTGA	ATCCCAACAC	CATGGGAGGC	4620
TGAGATGGGT	GGATCACGAG	GTCAGGAGTT	TGAGACCAGC	CTGACCAACA	TGGTGAAACT	4680
CTGTCTCTAC	TAAAAAAGAA	AAAAATAGAA	AAATTAGCCG	GGCGTGGTGG	CACACGGCAC	4740
CTGTAATCCC	AGCTACTGAG	GAGGCTGAGG	CAGGAGAATC	ACTTGAACCC	AGAAGGCAGA	4800
GGTTGCAATG	AGCCGAGATT	GCGCCACTGC	ACTCCAGCCT	GGGTGACAGA	GTGAGACTCT	4860
GTCTCAAAAA	AAAAAAATTT	TTTTTTTTTT	TTTGTAGAGA	TGGATCTTGC	TTTGTCTCTC	4920
TGGTTGGCCT	TGAACCTCTG	GCTTCAAGTG	ATCCTCCTAC	CTTGGCCTCG	GAAAGTGTGT	4980
GGATTACAGG	CGTGAGCCAC	CATGACTGAC	CTGTCTGTAA	TCTTGAGGTA	CATAAACCTG	5040
GCTCCTAAAG	GCTAAAGGCT	AAATATTTGT	TGGAGAAGGG	GCATTGGATT	TTGCATGAGG	5100
ATGATTCTGA	CCTGGGAGGG	CAGGTGAGCA	GGCATCTCTG	TTGCACAGAT	AGAGTGTACA	5160
GGTCTGGAGA	ACAAGGAGTG	GGGGGTTATT	GGAATTCAC	ATTGTTTGCT	GCACGTTGGA	5220
TTTTGAAATG	CTAGGGAAC	TTGGGAGACT	CATATTTCTG	GGCTAGAGGA	TCTGTGGACC	5280

ACAAGATCTT	TTTATGATGA	CAGTAGCAAT	GTATCTGTGG	AGCTGGATTC	TGGGTTGGGA	5340
GTGCAAGGAA	AAGAAATGTAC	TAAATGCCAA	GACATCTATT	TCAGGAGCAT	GAGGAATAAA	5400
AGTTCTAGTT	TCTGGTCTCA	GAGTGGTGCA	GGGATCAGGG	AGTCTCACAA	TCTCCTGAGT	5460
GCTGGTGTCT	TAGGGCACAC	TGGGTCTTGG	AGTGCAAAGG	ATCTAGGCAC	GTGAGGCTTT	5520
GTATGAAGAA	TCGGGGATCG	TACCCACCCC	CTGTTTCTGT	TTCATCCTGG	GCATGTCTCC	5580
TCTGCCTTTG	TCCCCTAGAT	GAAGTCTCCA	TGAGCTACAA	GGGCCTGGTG	CATCCAGGGT	5640
GATCTAGTAA	TTGCAGAACA	GCAAGTGCTA	GCTCTCCCTC	CCCTTCCACA	GCTCTGGGTG	5700
TGGGAGGGGG	TTGTCCAGCC	TCCAGCAGCA	TGGGGAGGGC	CTTGGTCAGC	CTCTGGGTGC	5760
CAGCAGGGCA	GGGGCGGAGT	CCTGGGGAAT	GAAGGTTTTA	TAGGGCTCCT	GGGGGAGGCT	5820
CCCCAGCCCC	AAGCTT					5836

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5835 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGCTTCTAG	TTTTCTTTTC	CCGGTGACAT	CGTGGAAAGC	ACTAGCATCT	CTAAGCAATG	60
ATCTGTGACA	ATATTACAG	TGTAATGCCA	TCCAGGGAAC	TCAACTGAGC	CTTGATGTCC	120
AGAGATTTTT	GTGTTTTTTT	CTGAGACTGA	GTCTCGCTCT	GTGCCAGGCT	GGAGTGCAGT	180
GGTGCAACCT	TGGCTCACTG	CAAGCTCCGC	CTCCTGGGTT	CACGCCATTG	TCCTGCCTCA	240
GCCTCCTGAG	TAGCTGGGAC	TACAGGCACC	CGCCACCACG	CCTGGCTAAT	TTTTTTGTAT	300
TTTTAGTAGA	GATGGGGTTT	CACGTGTGTTA	GCCAGGATGG	TCTCAGTCTC	CTGACCTCGT	360
GATCTGCCCC	CCTTGGCCTC	CCAAAGTGCT	GGGATGACAG	GCGTGAGCCA	CCGCGCCTGG	420
CCGATATCCA	GAGATTTTTT	GGGGGGCTCC	ATCACACAGA	CATGTTGACT	GTCTTCATGG	480
TTGACTTTTA	GTATCCAGCC	CCTCTAGAAA	TCTAGCTGAT	ATAGTGTGGC	TCAAAACCTT	540
CAGCAGAAAT	CACACCGTTA	GACTATCTGG	TGTGGCCCCA	ACCTTCAGGT	GAACAAAGGG	600
ACTCTAATCT	GGCAGGATAC	TCCAAAGCAT	TAGAGATGAC	CTCTTGCAAA	GAAGAAAGAA	660
TGGAAAAGAA	AAAGAAAGAA	AGGAAAAAAA	AAAAAAAATA	GAGATGACCT	CTCAGGCTCT	720
GAGGGGAAAC	GCCTGAGGTC	TTTGAGCAAG	GTCAGTCCTC	TGTTGCACAG	TCTCCCTCAC	780
AGGGTCATTG	TGACGATCAA	ATGTGGTCAC	GTGTATGAGG	CACCAGCACA	TGCCTGGCTC	840
TGGGGAGTGC	CGTGTAAGTG	TATGCTTGCA	CTGCTGAATG	GCTGGGATGT	GTCAGGGATT	900
ATCTTCAGCA	CTTACAGATG	CTCATCTCAT	CCTCACAGCA	TCACTATGGG	ATGGGTATTA	960
CTGGCCTCAT	TTGATGGAGA	AAGTGGCTGT	GGCTCAGAAA	GGGGGGACCA	CTAGACCAGG	1020
GACACTCTGG	ATGCTGGGGA	CTCCAGAGAC	CATGACCACT	CACCAACTGC	AGAGAAATTA	1080
ATTGTGGCCT	GATGTCCCTG	TCCTGGAGAG	GGTGGAGGTG	GACCTTCACT	AACCTCCTAC	1140
CTTGACCCTC	TCTTTTAGGG	CTCTTTCTGA	CCTCCACCAT	GGTACTAGGA	CCCCATTGTA	1200
TTCTGTACCC	TCTTGACTCT	ATGACCCCCA	CCGCCCCTG	CATCCAGCTG	GGTCCCCTCC	1260
TATCTCTATT	CCCAGCTGGC	CAGTGCAGTC	TCAGTGCCCA	CCTGTTTGTC	AGTAACCTG	1320
AAGGGGCTGA	CATTTTACTG	ACTTGCAAAC	AAATAAGCTA	ACTTTCCAGA	GTTTTGTGAA	1380
TGCTGGCAGA	GTCCATGAGA	CTCCTGAGTC	AGAGGCAAAG	GCTTTTACTG	CTCACAGCTT	1440
AGCAGACAGC	ATGAGGTTCA	TGTTACATT	AGTACACCTT	GGCCCCCCCC	AATCTTGTAG	1500
GGTGACCAGA	GCAGTCTAGG	TGGATGCTGT	GCAGAAGGGG	TTTGTGCCAC	TGGTGAGAAA	1560
CCTGAGATTA	GGAAATCCTCA	ATCTTATACT	GGGACAACCT	GCAAACCTGC	TCAGCCTTTG	1620
TCTCTGATGA	AGATATTATC	TTCATGATCT	TGGATTGAAA	ACAGACCTAC	TCTGGAGGAA	1680
CATATTGTAT	CGATTGTCTT	TGACAGTAAA	CAAATCTGTT	GTAAGAGACA	TTATCTTTAT	1740
TATCTAGGAC	AGTAAGCAAG	CCTGGATCTG	AGAGAGATAT	CATCTTGCAA	GGATGCCTGC	1800
TTTACAAACA	TCCTTGAAAC	AACAATCCAG	AAAAAAAAG	GTGTTACTGT	CTTTGCTCAG	1860
AAGACACACA	GATACGTGAC	AGAACCATGG	AGAATTGCCT	CCCAACGCTG	TTCAGCCAGA	1920
GCCTTCCACC	CTTTCTGCAG	GACAGTCTCA	ACGTTCCACC	ATTAAATACT	TCTTCTATCA	1980
CATCCCGCTT	CTTTATGCCT	AACCAAGGTT	CTAGGTCCCG	ATCGACTGTG	TCTGGCAGCA	2040

CTCCACTGCC	AAACCCAGAA	TAAGGCAGCG	CTCAGGATCC	CGAAGGGGCA	TGGCTGGGGA	2100
TCAGAACTTC	TGGGTTTGAG	TGAGGAGTGG	GTCCACCCTC	TTGAATTTCA	AAGGAGGAAG	2160
AGGCTGGATG	TGAAGGTACT	GGGGGAGGGA	AAGTGTCACT	TCCGAACCTC	TAGGTCAATG	2220
AGGGAGGAGA	CTGGTAAGGT	CCCAGCTCCC	GAGGTACTGA	TGTGGGAATG	GCCTAAGAAT	2280
CTCATATCCT	CAGGAAGAAG	GTGCTGGAAT	CGTGAGGGGT	AGAGTTCTGG	GTATATTTGT	2340
GGCTTAAGGC	TCTTTGGCCC	CTGAAGGCAG	AGCTGGGAAC	CATTAGGTCC	AGGGTTTGGG	2400
GTGATAGTAA	TGGGATCTCT	TGATTCTCTA	AGAGTCTGAG	GATCGAGGGT	TGCCCATTCT	2460
TCCATCTTGC	CACCTAATCC	TTACTCCACT	TGAGGGTATC	ACCAGCCCTT	CTAGCTCCAT	2520
GAAGGTCCCC	TGGGCAAGCA	CAATCTGAGC	ATGAAAGATG	CCCCAGAGGC	CTTGGGTGTC	2580
ATCCACTCAT	CATCCAGCAT	CACACTCTGA	GGGTGTGGCC	AGCACCATGA	CGTCATGTTG	2640
CTGTGACTAT	CCCTGCAGCG	TGCCTCTCCA	GCCACCTGCC	AACCGTAGAG	CTGCCCATCC	2700
TCCTCTGGTG	GGAGTGGCCT	GCATGGTGCC	AGGCTGAGGC	CTAGTGTGAG	ACAGGGAGCC	2760
TGGAATCATA	GGGATCCAGG	ACTCAAAAGT	GCTAGAGAAT	GGCCATATGT	CACCATCCAT	2820
GAAATCTCAA	GGGCTTCTGG	GTGGAGGGCA	CAGGGACCTG	AACTTATGGT	TTCCCAAGTC	2880
TATTGCTCTC	CCAAGTGAGT	CTCCAGATA	CGAGGCACATG	TGCCAGCATC	AGCCTTATCT	2940
CCACCACATC	TTGTAAAAGG	ACTACCCAGG	GCCCTGATGA	ACACCATGGT	GTGTACAGGA	3000
GTAGGGGGTG	GAGGCACGGA	CTCCTGTGAG	GTCACAGCCA	AGGGAGCATC	ATCATGGGTG	3060
GGGAGGAGGC	AATGGACAGG	CTTGAGAACG	GGGATGTGGT	TGTATTTGGT	TTTCTTTGGT	3120
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TCAGATTGGA	GTTGGGTTAG	ATAAAGTGCT	GGGTATAGGA	TTGAGAGTGG	AGTATGAAGA	3240
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GATGAGTTTG	GGATTGACAC	TGTGGAGGTG	GTTTGGGATG	GCATGGCTTT	GGGATGGAAA	3360
TAGATTTGTT	TTGATGTTGG	CTCAGACATC	CTTGGGGATT	GAAGTGGGGA	TGAAGCTGGG	3420
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CTCACTGCAA	CCTCTGCCTC	CCAGGTTCAA	GTGATTCTCC	TGCCTCAGCC	TCCTGAGTTG	4020
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GGTTTCACCA	ATGTTTGCCA	GGCTGGCCTC	GAACTCTGA	CCTGGTGATC	CACCTGCCTC	4140
AGCCTCCCAA	AGTGCTGGGA	TTACAGGCGT	CAGCCACCGC	GCCCAGCCAC	TTTTGTCAAA	4200
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CTCTTAAGAG	TTCCCGATTG	TCTTCTGAGA	CTACAAATTG	TGATTTTGCA	TGCCACCTTA	4380
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GCTTCGGTCT	CCCAATAGCT	AAGACTACAG	TAGTCCACCA	CCATATCCAG	ATAATTTTTA	4560
AATTTTTTGG	GGGGCCGGGC	ACAGTGGCTC	ACGCCTGTAA	TCCCAACACC	ATGGGAGGCT	4620
GAGATGGGTG	GATCACGAGG	TCAGGAGTTT	GAGACCAGCC	TGACCAACAT	GGTGAAACTC	4680
TGTCTCTACT	AAAAAAAAAA	AAATAGAAA	AATTAGCCGG	GCGTGGTGGC	ACACGGCACC	4740
TGTAATCCCA	GCTACTGAGG	AGGCTGAGGC	AGGAGAATCA	CTTGAACCCA	GAAGGCAGAG	4800
GTTGCAATGA	GCCGAGATTG	CGCCACTGCA	CTCCAGCCTG	GGTGACAGAG	TGAGACTCTG	4860
TCTCAAAAAA	AAAAAATTTT	TTTTTTTTTT	TTGTAGAGAT	GGATCTTGCT	TTGTTTCTCT	4920
GGTTGGCCTT	GAACCTCTGG	CTTCAAGTGA	TCCTCCTACC	TTGGCCTCGG	AAAGTGTTGG	4980
GATTACAGGC	GTGAGCCACC	ATGACTGACC	TGTCGTAAAT	CTTGAGGTAC	ATAAACCTGG	5040
CTCCTAAAGG	CTAAAGGCTA	AATATTTGTT	GGAGAAGGGG	CATTGGATTT	TGCATGAGGA	5100
TGATTCTGAC	CTGGGAGGGC	AGGTCAGCAG	GCATCTCTGT	TGCACAGATA	GAGTGTACAG	5160
GTCTGGAGAA	CAAGGAGTGG	GGGGTTATTG	GAATTCACAC	TTGTTTGCTG	CACGTTGGAT	5220
TTTGAAATGC	TAGGGAACCT	TGGGAGACTC	ATATTTCTGG	GCTAGAGGAT	CTGTGGACCA	5280
CAAGATCTTT	TTATGATGAC	AGTAGCAATG	TATCTGTGGA	GCTGGATTCT	GGGTTGGGAG	5340
TGCAAGGAAA	AGAATGTACT	AAATGCCAAG	ACATCTATTT	CAGGAGCATG	AGGAATAAAA	5400

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GTTCTAGTTT CTGGTCTCAG AGTGGTGCAT GGATCAGGGA GTCTCACAAT CTCCTGAGTG 5460
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TATGAAGAAT CGGGGATCGT ACCCACCCCC TGTTCCTGTT TCATCCTGGG CATGTCTCCT 5580
CTGCCTTTGT CCCCTAGATG AAGTCTCCAT GAGCTACAAG GGCCTGGTGC ATCCAGGGTG 5640
ATCTAGTAAT TGCAGAACAG CAAGTGCTAG CTCTCCCTCC CCTTCCACAG CTCTGGGTGT 5700
GGGAGGGGGT TGTCCAGCCT CCAGCAGCAT GGGGAGGGCC TTGGTCAGCC TCTGGGTGCC 5760
AGCAGGGCAG GGGCGGAGTC CTGGGGAATG AAGGTTTAT AGGGCTCCTG GGGGAGGCTC 5820
CCCAGCCCCA AGCTT 5835

```

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCACCGGT AAGCTTCCAC AAGTGCATTT AGCC 34

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCACCGGT CTGTAGGTAT CTGGACCTCA CTG 33

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCCGGCCG AAGCTTCCAC AAGTGCATTT AGCC 34

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCGGCCG CTGTAGGTAT CTGGACCTCA CTG

33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCGTCTTCAA GAATTCTCA

19

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTCAGTCAC CGGTGTCGGA

20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCATTCTCTA GACACAGGTG

20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCCGACACCG GTGACTGAAA

20

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCCACGGCC GCATTATATA C

21

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTATATAATG CGGCCGTGGG C

21

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCAGAAAATC CAGCAGGTAC C

21

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCTTAATTA AAAGCAAACC TCACCTCCG

29



## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTGGAACAAA AGGTGATTAA AAAATCCCAG

30

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACCTTTTGT TCCACCGCTC TGCTTATTAC

30

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGCTTAATTA ACTGTGAAAG GTGGGAGC

28

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCAGCTCACT TAAGTTCATG TCG

23

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCAGCCTAGG AAATATGACT ACGTCCG

27

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 2...304
- (D) OTHER INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

G ATG ACC GGC TCA ACC ATC GCG CCC ACA ACG GAC TAT CGC AAC ACC ACT	49
Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr Thr	
1 5 10 15	
GCT ACC GGA CTA ACA TCT GCC CTA AAT TTA CCC CAA GTT CAT GCC TTT	97
Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe	
20 25 30	
GTC AAT GAC TGG GCG AGC TTG GAC ATG TGG TGG TTT TCC ATA GCG CTT	145
Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu	
35 40 45	
ATG TTT GTT TGC CTT ATT ATT ATG TGG CTT ATT TGT TGC CTA AAG CGC	193
Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg	
50 55 60	
AGA CGC GCC AGA CCC CCC ATC TAT AGG CCT ATC ATT GTG CTC AAC CCA	241
Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro	
65 70 75 80	
CAC AAT GAA AAA ATT CAT AGA TTG GAC GGT CTG AAA CCA TGT TCT CTT	289
His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser Leu	
85 90 95	
CTT TTA CAG TAT GAT TAA	307
Leu Leu Gln Tyr Asp	
100	

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 101 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```

Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr Thr
 1           5           10           15
Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe
      20           25           30
Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu
      35           40           45
Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg
      50           55           60
Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro
      65           70           75           80
His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser Leu
      85           90           95
Leu Leu Gln Tyr Asp
      100

```

## CLAIMS

What is claimed is:

5

1. An adenovirus vector comprising an adenovirus gene under transcriptional control of a probasin transcriptional regulatory element (PB-TRE).

2. The adenovirus vector of claim 1, wherein the adenovirus gene is essential for viral replication.

10

3. The adenovirus vector of claim 2, wherein the adenovirus gene is an early gene.

4. The adenovirus of claim 2, wherein the adenovirus gene is a late gene.

5. The adenovirus vector of claim 3, wherein the adenovirus early gene is E1A.

6. The adenovirus vector of claim 3, wherein the adenovirus early gene is E1B.

15

7. The adenovirus vector of claim 1, wherein the adenovirus gene is the adenovirus death protein gene (ADP).

8. The adenovirus vector of claim 1, wherein the PB-TRE comprises an enhancer from a probasin gene.

9. The adenovirus vector of claim 1, wherein the PB-TRE comprises a promoter from a probasin gene.

20

10. The adenovirus vector of claim 1, wherein the PB-TRE comprises a promoter from a probasin gene and an enhancer from a probasin gene.

11. The adenovirus vector of claim 1, wherein the PB-TRE comprises at least one androgen response element (ARE).

12. The adenovirus vector of claim 1, wherein the PB-TRE comprises androgen response elements 1 and 2 (ARE-1 and ARE-2) of a probasin gene.
13. The adenovirus vector of claim 1, wherein the PB-TRE comprises nucleotides about 141 to about 454 of SEQ ID NO:1.
- 5        14. The adenovirus vector of claim 1, wherein the PB-TRE comprises nucleotides about 191 to about 204 or about 286 to about 310 of SEQ ID NO:1.
15. The adenovirus vector of claim 1, wherein the PB-TRE comprises nucleotides about 191 to about 204 and about 286 to about 310 of SEQ ID NO:1.
- 10       16. The adenovirus vector of claim 1, wherein the PB-TRE comprises the sequence of SEQ ID NO:1.
17. A composition comprising an adenovirus of claim 1.
18. A composition of claim 17, further comprising a pharmaceutically acceptable excipient.
- 15       19. The adenovirus of claim 1, further comprising at least one additional adenovirus gene under transcriptional control of at least one additional prostate-specific transcriptional regulatory element.
20. The adenovirus of claim 19, wherein the at least one additional prostate-specific transcriptional regulatory element comprises a PB-TRE.
- 20       21. The adenovirus of claim 19, wherein the at least one additional prostate-specific transcriptional regulatory element comprises a prostate-specific antigen (PSA) transcriptional regulatory element.
22. A composition comprising an adenovirus of claim 19.

23. The composition of claim 22, further comprising a pharmaceutically acceptable excipient.

24. An adenovirus vector of claim 1, further comprising a heterologous gene under transcriptional control of a probasin transcriptional regulatory element (PB-TRE).

5        25. The vector of claim 24, wherein the heterologous gene is a reporter gene.

26. The vector of claim 24, wherein the heterologous gene is conditionally required for cell survival.

27. A host cell transformed with an adenovirus vector of claim 1.

28. A host cell transformed with an adenovirus vector of claim 19.

10       29. A method of detecting cells which allow a PB-TRE to function in a biological sample comprising the steps of:

contacting a biological sample with an adenovirus vector of claim 1, under conditions suitable for PB-TRE-mediated gene expression in cells which allow a PB-TRE to function; and

15       determining if PB-TRE mediates gene expression in the biological sample,

wherein PB-TRE-mediated gene expression is indicative of the presence of cells which allow a PB-TRE to function.

30. The method of claim 29, in which cells which allow a PB-TRE to function express androgen receptor.

20       31. A method of propagating an adenovirus specific for cells which allow a PB-TRE to function, said method comprising:

combining an adenovirus according to claim 1 with cells which allow a PB-TRE to function,

whereby said adenovirus is propagated.

32. A method of propagating an adenovirus specific for mammalian cells which allow  
5 a PB-TRE to function, said method comprising:

combining an adenovirus according to claim 19 with mammalian cells which allow a PB-TRE to function,

whereby said adenovirus is propagated.

33. A method for modifying the genotype of a target cell, said method comprising  
10 contacting a cell which allows a PB-TRE to function with an adenovirus of claim 1, wherein the adenovirus enters the cell.

34. A method for modifying the genotype of a target cell, said method comprising contacting a cell which allows a PB-TRE to function with an adenovirus of claim 19, wherein the adenovirus enters the cell.

15 35. A method for conferring selective cytotoxicity on a target cell, said method comprising contacting a cell which allows a PB-TRE to function with an adenovirus vector of claim 1, wherein the adenovirus vector enters the cell.

36. A method for conferring selective toxicity on a target cell, said method comprising contacting a cell which allows a PB-TRE to function with an adenovirus vector of claim 19,  
20 wherein the adenovirus vector enters the cell.

37. A method of suppressing tumor cell growth, said method comprising contacting a tumor cell with an adenoviral vector of claim 1 such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

38. A method of suppressing tumor cell growth, said method comprising contacting a tumor cell with an adenoviral vector of claim 19 such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

5 39. A method of treating prostate cancer in an individual, comprising the step of administering to the individual an effective amount of an adenovirus vector of claim 2.

40. An adenovirus comprising an adenoviral vector of claim 1, wherein the adenovirus is complexed with a masking agent.

41. The adenovirus of claim 40 wherein the masking agent is polyethyleneglycol (PEG).

10 42. The adenovirus of claim 41, wherein the PEG is of a molecular weight between about 2500 to about 30,000.

43. The adenovirus of claim 42, wherein the PEG is of a molecular weight between about 3000 to about 20,000.

15 44. The adenovirus of claim 43, wherein the PEG is of a molecular weight between about 5000 to about 10,000.

45. The adenovirus of claim 41, wherein the PEG is covalently attached to the adenovirus.

46. The adenovirus of claim 41, wherein the PEG is non-covalently attached to the adenovirus.

20 47. The adenovirus of claim 44, wherein the PEG is covalently attached by using a N-hydroxysuccinimidyl (NHS) active ester.

48. The adenovirus of claim 46, wherein the N-hydroxysuccinimidyl (NHS) active ester is selected from the group consisting of succinimidyl succinate, succinimidyl succinamide and succinimidyl propionate.

25 49. The adenovirus of claim 48, wherein the N-hydroxysuccinimidyl (NHS) active ester is succinimidyl succinate.



50. A method of making a masked adenovirus, comprising covalently attaching a masking agent to an adenovirus, wherein the masking agent has a molecular weight between about 2500 and about 20,000, thereby producing a masked adenovirus.

51. The method of claim 50, wherein the masking agent is polyethyleneglycol (PEG).

52. An adenovirus complexed with a masking agent.

53. The adenovirus of claim 52 wherein the masking agent is polyethyleneglycol (PEG).

54. The adenovirus of claim 53, wherein the PEG is of a molecular weight between about 2500 to about 30,000.

55. The adenovirus of claim 54, wherein the PEG is of a molecular weight between about 3000 to about 20,000.

56. The adenovirus of claim 55, wherein the PEG is of a molecular weight between about 5000 to about 10,000.

57. The adenovirus of claim 53, wherein the PEG is covalently attached to the adenovirus.

58. The adenovirus of claim 53, wherein the PEG is non-covalently attached to the adenovirus.

59. The adenovirus of claim 57, wherein the PEG is covalently attached by using a N-hydroxysuccinimidyl (NHS) active ester.

60. The adenovirus of claim 59, wherein the N-hydroxysuccinimidyl (NHS) active ester is selected from the group consisting of succinimidyl succinate, succinimidyl succinamide and succinimidyl propionate.






61. The adenovirus of claim 60, wherein the N-hydroxysuccinimidyl (NHS) active ester is succinimidyl succinate.

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-426  
5'—AAGCTTCCACAAGTGCATTTAGCCTCTCCAGTATTGCTGATGAATCCACAGT  
TCAGGTTCAATGGCGTTCAAACTTGATCAAAAATGACCAGACTTTATATTCTTA  
CACCAACATCTATCTGATTGGAGGAATGGATAATAGTCATCATGTTTAAACATCT  
ACCATTCCAGTTAAGAAAATATGATAGCATCTTGTTCTTAGTCTTTTTCTTAATA  
ARE-1  
GGGACATAAAGCCCAAAATAAAAATATGCCTGAAGAATGGGACAGGCATTGG  
GCATTGTCCATGCCTAGTAAAGTACTCCAAGAACCTATTTGTATACTAGATGACA  
ARE-2  
CAATGTCAATGTCTGTGTACAACCTGCCAACTGGGATGCAAGACACTGCCCCATG  
CCAATCATCCTGAAAAGCAGCTATAAAAGCAGGAAGCTACTCTGCACCTT  
CAAT BOX TATAA BOX TRANSCRIPTION SITE  
+1  
+28  
GTCAGTGAGGTCCAGATACCTACAG—3'

FIG. 1

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 PBE (RAT PROBASIN PROMOTER/ ENHANCER)  
 PSE  
 FIREFLY LUCIFERASE GENE (LUC)  
 SV40 ENHANCER  
 SV40 LATE POLY(A) SIGNAL

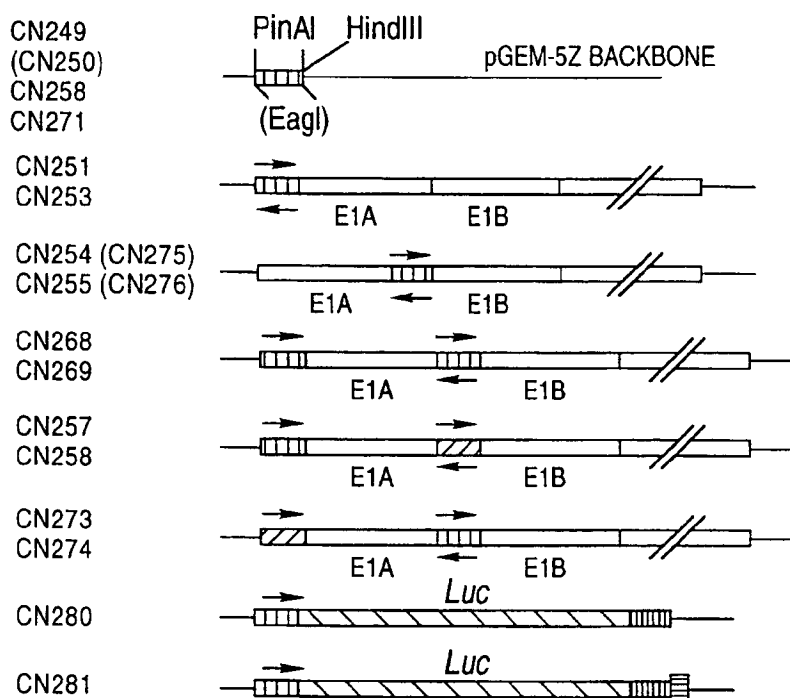


FIG. 2A

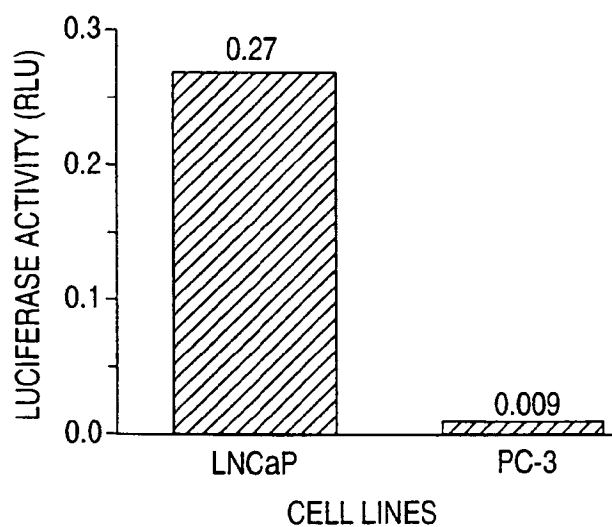
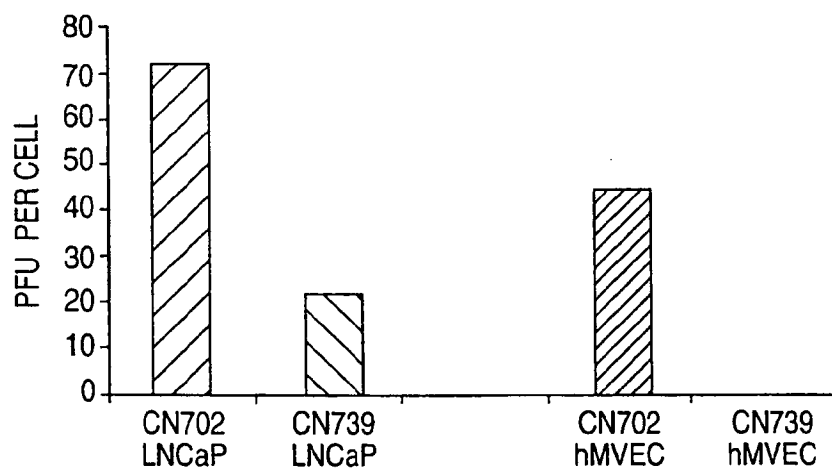
3/12



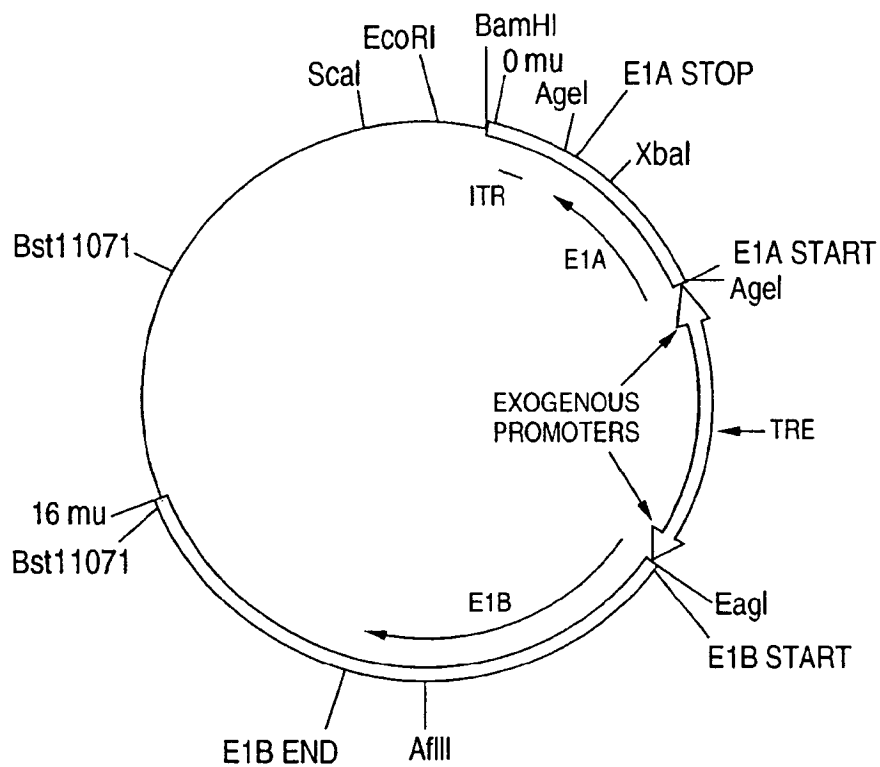
FIG. 2B

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**FIG. 3****FIG. 6**

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**FIG. 4**

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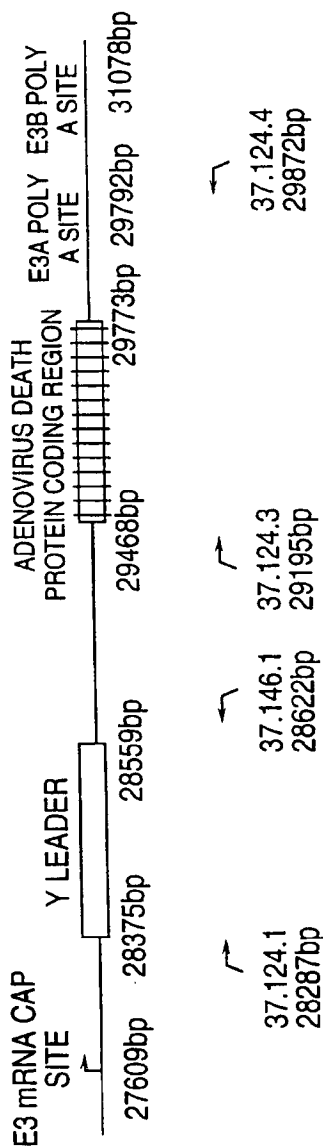


FIG. 5A

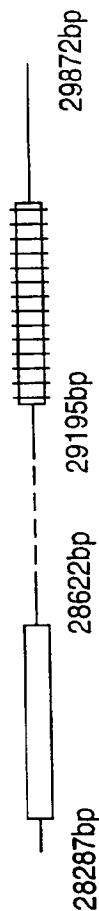


FIG. 5B

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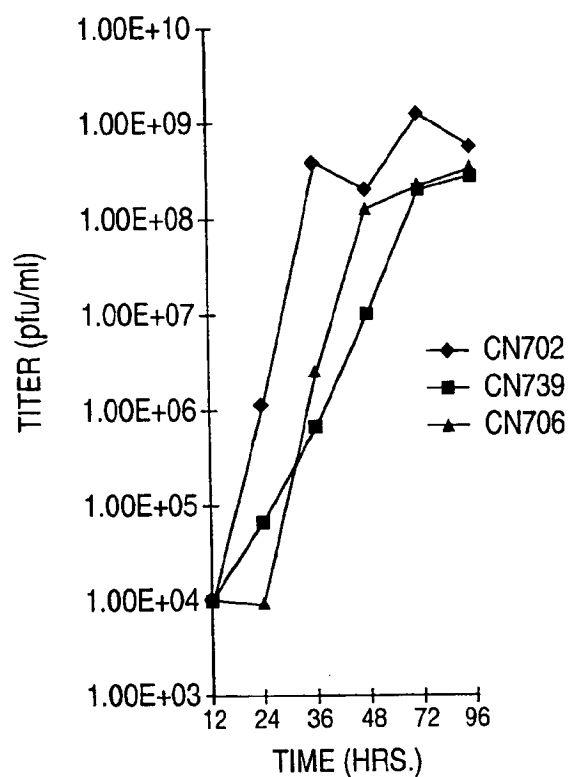


FIG. 7A

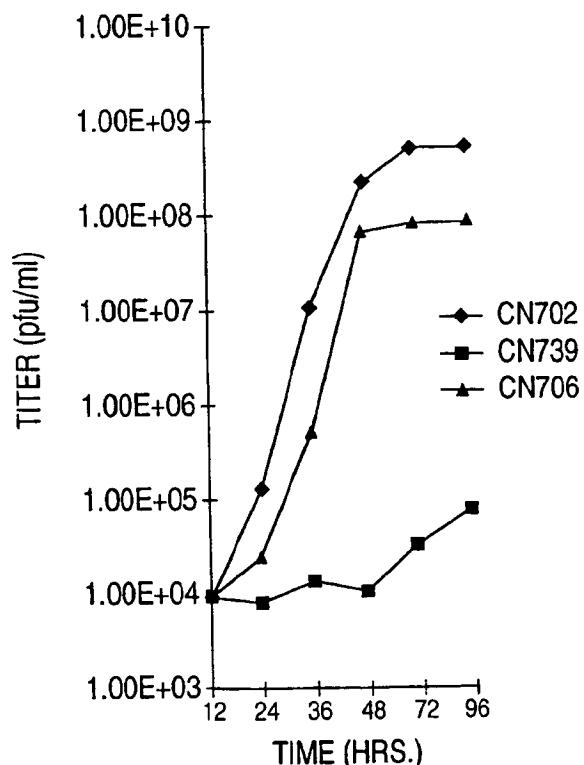


FIG. 7B

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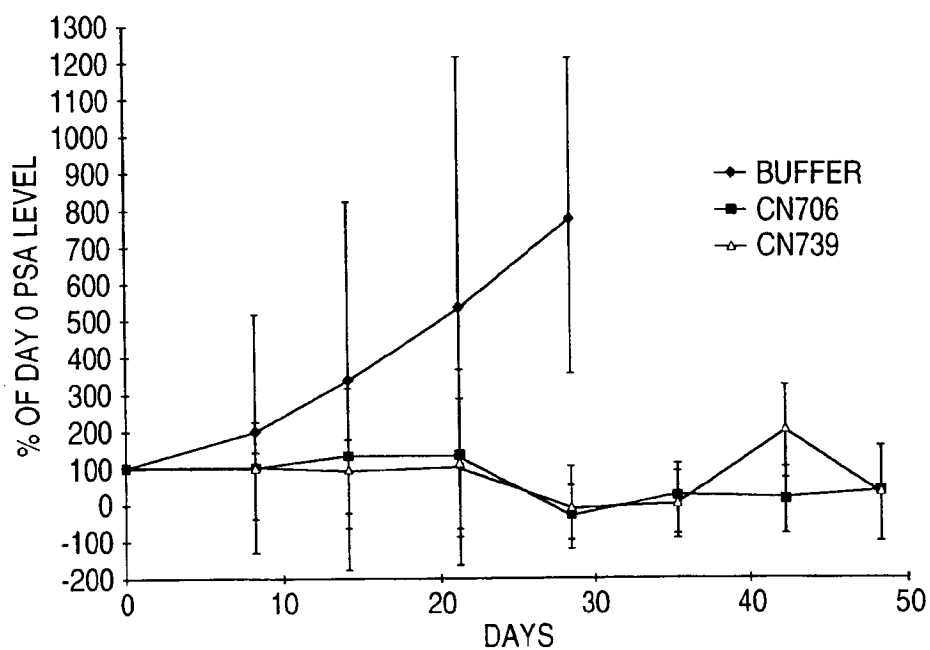


FIG. 8

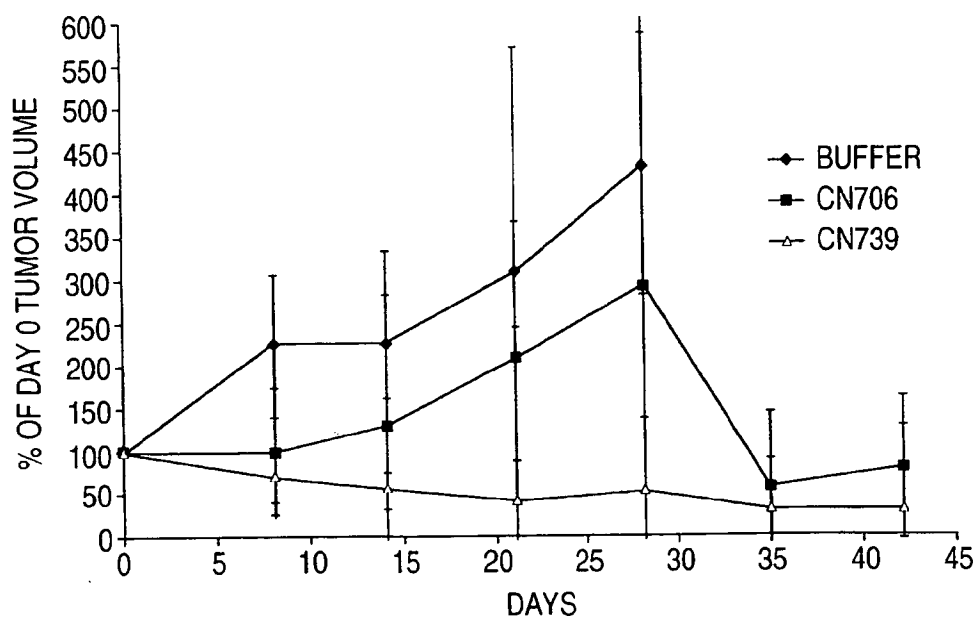


FIG. 9

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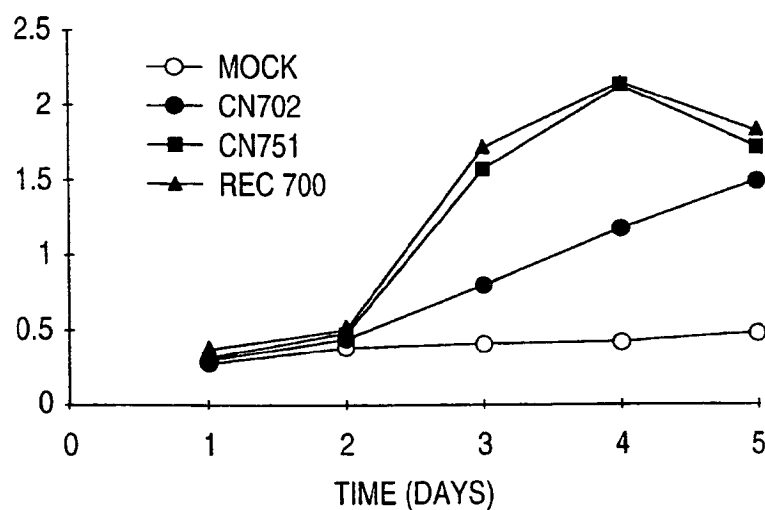


FIG. 10

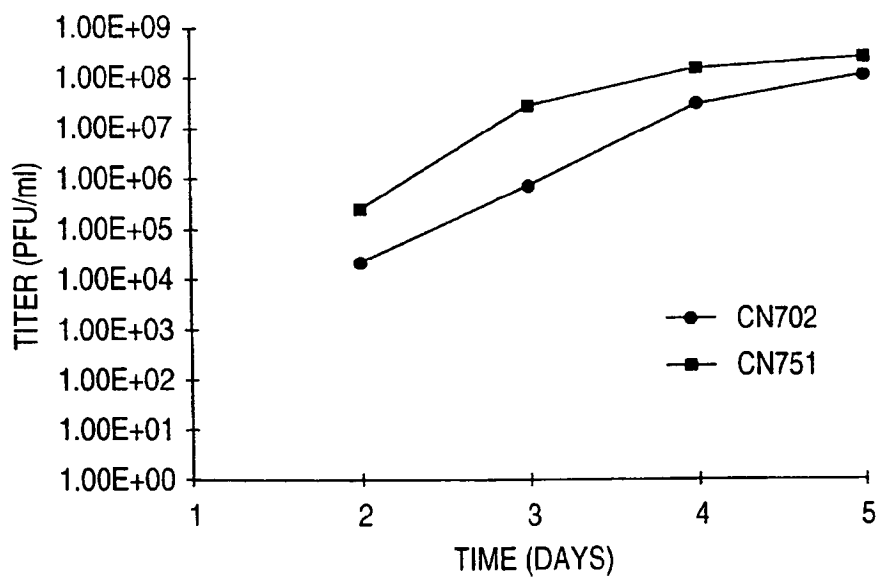


FIG. 11

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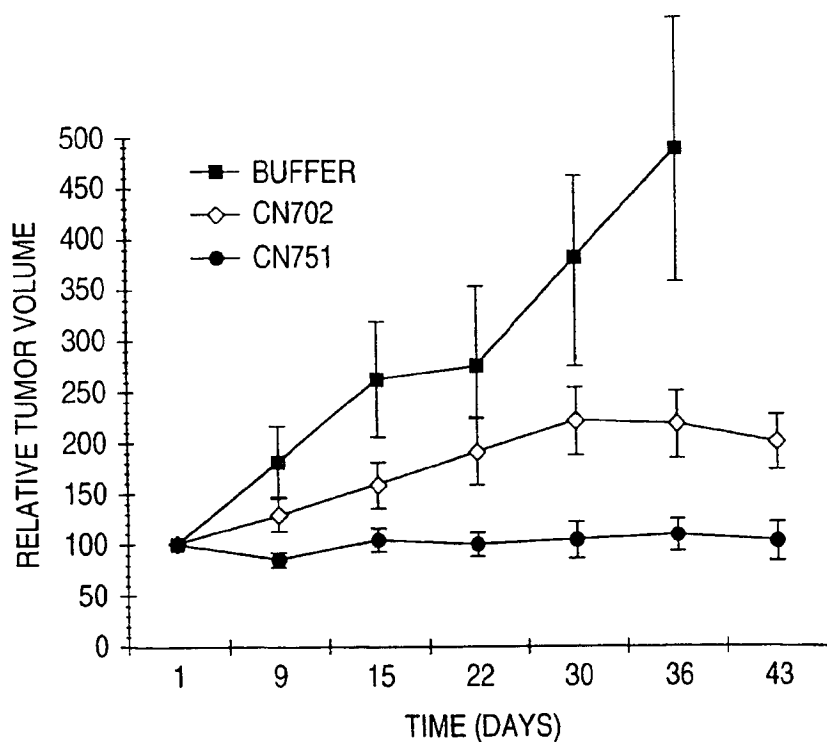


FIG. 12

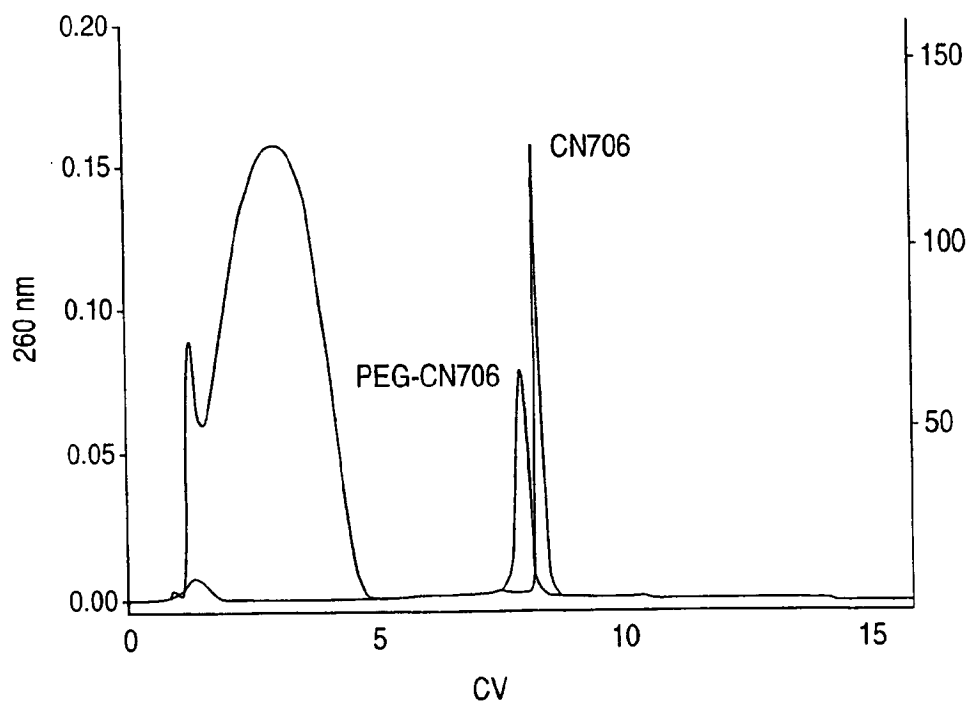


FIG. 15

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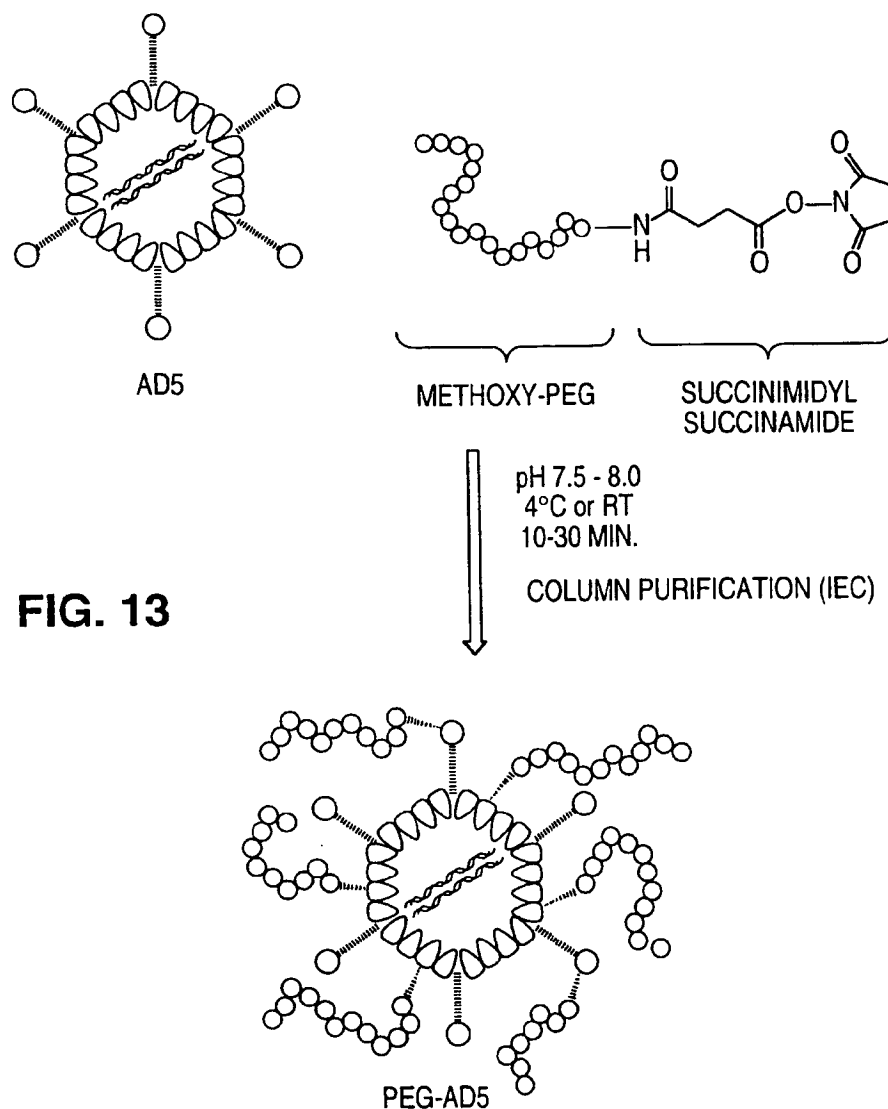


FIG. 13

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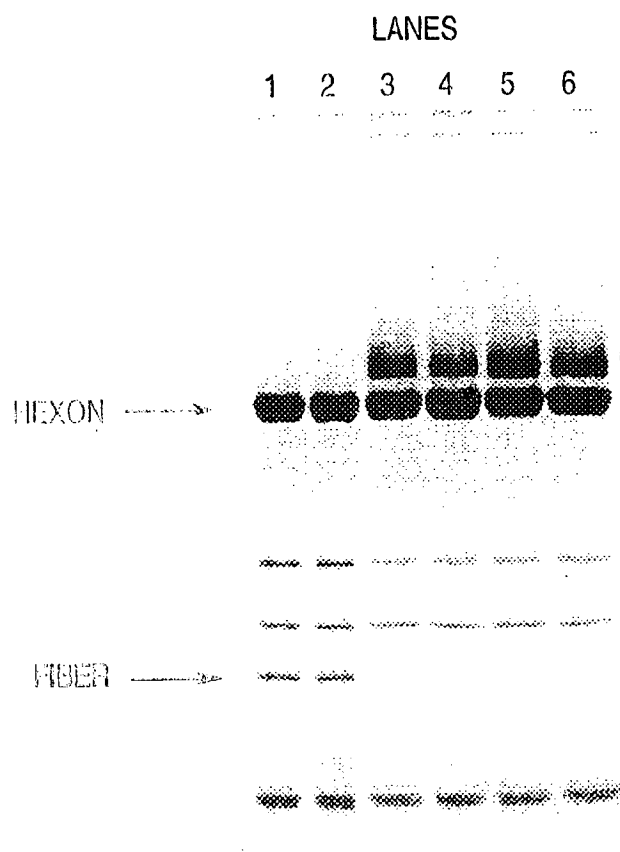


FIG. 14

**SUBSTITUTE SHEET (RULE 26)**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT) -

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/86, 5/10, A61K 48/00, 47/48, C12Q 1/70, C12N 11/08</b>		<b>A3</b>	(11) International Publication Number: <b>WO 98/39466</b> (43) International Publication Date: 11 September 1998 (11.09.98)
(21) International Application Number: <b>PCT/US98/04132</b> (22) International Filing Date: 3 March 1998 (03.03.98) (30) Priority Data: 60/039,762 3 March 1997 (03.03.97) US 09/033,333 2 March 1998 (02.03.98) US (71) Applicant (for all designated States except US): CALYDON, INC. [US/US]; 1324 Chesapeake Terrace, Sunnyvale, CA 94089 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): YU, De-Chao [CN/US]; 1046 Eagle Lane, Foster City, CA 94404 (US). HENDERSON, Daniel, R. [US/US]; 955 Matadero Avenue, Palo Alto, CA 94306 (US). SCHUUR, Eric, R. [US/US]; 20350 Stevens Creek Boulevard #305, Cupertino, CA 95014 (US). LAMPARSKI, Henry, G. [US/US]; 422 South El Dorado, San Mateo, CA 94402 (US). (74) Agents: POLIZZI, Catherine, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> (88) Date of publication of the international search report: 14 January 1999 (14.01.99)	
(54) Title: <b>ADENOVIRUS VECTORS SPECIFIC FOR CELLS EXPRESSING ANDROGEN RECEPTOR AND METHODS OF USE THEREOF</b>			
(57) Abstract <p>Replication-competent adenovirus vectors specific for cells which allow a probasin transcriptional response element (PB-TRE) to function, such as cells which express the androgen receptor (AR), and methods of use of such viruses are provided. These viruses comprise an adenoviral gene under control of a transcriptional regulatory portion of a PB-TRE, which is in turn dependent upon AR expression. The gene can be, for example, a gene required for viral replication or the adenovirus death protein gene (ADP). The viruses can also comprise at least one additional adenoviral gene under control of at least one additional prostate-specific transcriptional response element, such as that controlling prostate-specific antigen expression (PSA-TRE). Thus, virus replication can be restricted to target cells exhibiting prostate-specific gene expression, particularly prostate carcinoma cells. An adenovirus of the present invention can further comprise a heterologous gene such as a reporter under transcriptional control of a PB-TRE. The adenovirus vectors can be used to detect and monitor samples for the presence of prostate cells as well as to selectively kill malignant cells producing prostate-specific gene products.</p>			

\* (Referred to in PCT Gazette No. 49/1998, Section II)

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/04132

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N5/10 A61K48/00 A61K47/48 C12Q1/70  
C12N11/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 03594 A (UNIV MANITOBA ;MATUSIK ROBERT J (CA)) 17 February 1994 see the whole document ---	1-39
Y	GREENBERG N. M. ET AL.: "The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice." MOLECULAR ENDOCRINOLOGY, vol. 8, 1994, pages 230-239, XP002071135 cited in the application see the whole document ---	1-39
Y	WO 97 01358 A (CALYDON) 16 January 1997 see the whole document ---	1-39
-/--		

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

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\*Z\* document member of the same patent family

Date of the actual completion of the international search

10 July 1998

Date of mailing of the international search report

26.10.98

Name and mailing address of the ISA

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Mandl, B



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/04132

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 34969 A (CANJI INC) 7 November 1996 see the whole document ---	1-39
A	WO 96 17053 A (GENETIC THERAPY INC ;HALLENBECK PAUL L (US); CHANG YUNG NIEN (US);) 6 June 1996 see the whole document ---	1-39
A	TOLLEFSON A. E. ET AL.: "The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells." JOURNAL OF VIROLOGY, vol. 70, no. 4, April 1996, pages 2296-2306, XP002071004 see the whole document, especially page 2305, left column, last two lines and right column, first paragraph -----	7

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/04132

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 39 and claims 33-38, as far as in vivo application is concerned, are directed to a method of treatment of the human /animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see further information sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-49

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-49

An adenovirus vector comprising an adenovirus gene under transcriptional control of PB-TRE; a composition comprising said adenovirus vector; a host cell transformed with said adenovirus vector; a method for detecting cells which allow PB-TRE function; a method for propagating said adenovirus vector; a method for modifying the genotype of a target cell; a method for conferring selective cytotoxicity on a target cell; a method for suppressing tumor growth; and a method of treating prostate cancer.

2. Claims: 50-61

A method of making a masked adenovirus and an adenovirus masked with an masking agent.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/04132

PC1/US 96/04132

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9403594 A	17-02-94	AU 4694793 A	03-03-94
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		CA 2218390 A	07-11-96
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WO 9617053 A	06-06-96	AU 4504396 A	19-06-96
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		FI 972225 A	16-07-97
		NO 972373 A	25-07-97
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